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The role of RANTES in guinea pig inflammatory responses

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The role of RANTES in guinea pig inflammatory responses

Submitted by Emma Michelle Campbell
for the degree of Ph. D.
of the University of Bath.
1997

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Summary

RANTES is a member of the CC chemokine family and has previously been shown to attract human eosinophils, memory CD45RO T lymphocytes and monocytes; cells which comprise the cellular infiltrate in many allergic sites. Since the guinea pig is frequently used in the analysis of allergic inflammation, recombinant guinea pig (gp) RANTES was purified and characterised and patterns of RANTES mRNA expression in the lung determined, in order to assess its role in this species.

In a guinea pig model of allergic lung inflammation, no differences in RANTES mRNA were detected between naïve and ovalbumin sensitised animals after antigen challenge. In comparison, increased expression of MCP-1 mRNA was observed in sensitised animals compared to time-matched controls. *In situ* hybridisation analysis demonstrated that the macrophage was a potential source of RANTES, although bronchial epithelium and endothelial cells also stained positive. Furthermore, a similar cellular distribution was noted for eotaxin. However, neither RANTES nor eotaxin message were expressed in primary cultures of guinea pig lung fibroblasts, but these cells did express high levels of MCP-1 mRNA.

To investigate further the role of RANTES, the recombinant protein was purified and characterised. A high level of gpRANTES protein expression in *E. coli* was achieved by mutation of a human (h) RANTES expression construct to obtain a 68 amino acid protein identical to the predicted amino acid sequence, assuming an equivalent N terminus to hRANTES. Purified gpRANTES was an effective stimulus of human eosinophils as assessed by increases in intracellular free calcium in fura-2-loaded cells and chemotactic responses *in vitro*. gpRANTES exhibited similar potency and efficacy

to hRANTES. In marked contrast, neither gpRANTES nor hRANTES were able to activate guinea pig peritoneal eosinophils in these assays, even in the presence of priming agent, IL-5. However, gpRANTES was found to be a potent stimulator of guinea pig peritoneal macrophages. A similar profile of activity was noted *in vivo*. Following tracheal instillation of gpRANTES, a dose dependent increase in macrophage numbers but not eosinophils was observed in gpBAL. Macrophage accumulation was detectable by 6 h and sustained for at least 48 h. Furthermore, intradermal injection of gpRANTES into guinea pig skin sites induced the accumulation of macrophages at 24 h. However, eosinophils were not detected at either 2 or 24 h post injection. These results indicate that RANTES has a different cellular selectivity in the guinea pig than that described for the human, which may be important in the use of animal models in the analysis of allergic disorders.

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For Mum and Dad

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Publications resulting from this thesis

Campbell *et al* (1996) RANTES activates mononuclear cells but not eosinophils in the guinea pig. *Br. J. Pharmacol.* **119**, 50P (Abstract).

Campbell *et al* (1997) RANTES activates mononuclear cells but not eosinophils in the guinea pig. *J. Immunol.* In press.

Campbell *et al* (1997) Expression of RANTES and related chemokines in the guinea pig lung. In preparation.

Abbreviations

ANOVA	analysis of variance
APES	3-aminopropyltriethoxysaline
BAL	bronchoalveolar lavage
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BHR	bronchial hyperreactivity
bp	base pairs
BSA	bovine serum albumin
Bromophenol blue	3', 3'', 5', 5''- tetrabromophenolsulphonephthalein
C5a	complement fragment 5a
$[Ca^{2+}]_i$	intracellular free calcium concentration
CCR1-5	CC chemokine receptors 1-5
Con A	concanavalin A
CTAP-III	connective tissue activating protein III
Da/kDa	daltons/kilodaltons
DAG	diacylglycerol
DARC	Duffy antigen receptor
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphoshate
dGTP	deoxyguanosine 5'-triphoshate
dNTP	2' Deoxynucleoside 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
dUTP	deoxyuridine 5'-triphoshate
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DNase	deoxyribonuclease
DMEM	Dulbecco's minimum essential medium
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (b-aminoethylether) N, N, N', N'-tetraacetic acid
ELAM	endothelial-leukocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
ELR motif	tripeptide sequence of glutamic acid, leucine and arginine
ENA-78	epithelial derived neutrophil attractant-78
Endoproteinase Arg C	Endoproteinase Arginine C
EPO	eosinophil peroxidase
FEV_1	forced expiratory volume in one second
Fcα/ε/γR	Fc receptors for IgA, IgE, IgG
FCS	foetal calf serum
fMLP	N-formyl-Met-Leu-Phe

fura-2AM	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2 (2'-amino-5'-methylphenoxy)-ethane-N, N, N', N'-tetraacetic acid, pentaacetoxymethyl ester}
G-protein	GTP-binding protein
GMCSF	granulocyte-macrophage colony stimulating factor
gp	guinea pig
h	human
HBSS	Hanks' balanced salt solution
H&E	haematoxylin and eosin
HETE	hydroxyeicosatetraenoic acid
HRF	histamine releasing factors
ICAM-1/-2	intracellular adhesion molecule-1/-2
i.d.	intradermal
IFNγ	gamma interferon
Ig	immunoglobulin
IL-	interleukin-
IL-8-RA/B	IL-8 receptor A and B
^{125}I-HSA	^{125}Iodine-labelled human serum albumin
$^{111}In/^{111}InCl_3$	^{111}indium/indium chloride
i.m.	intramuscular
IMS	industrial methylated spirit
i.p.	intraperitoneal
IP_3	inositol 1, 4, 5-triphosphate
IPTG	isopropylthiogalactoside
IU	international units
LB medium	Luria Bertani medium
LT	leukotriene
LPR	late phase response
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic-activated cell sorter
MBP	major basic protein
MCP-1, 2, 3, 4	monocyte chemotactic peptide-1, 2, 3 and 4
MGSF	melanocyte growth stimulatory activity
MIP-1α, β	macrophage inflammatory protein-1 alpha/beta
M-MLV RTase	moloney murine leukemia virus reverse transcriptase
MØ	macrophage
mu	murine
n	number in study or group
N terminus	amino terminus
NAP-1, 2	neutrophil-activating protein-1, 2
NBT	nitroblue tetrazolium
OD	optical density
OVA	ovalbumin
p	probability
PAGE	polyacrylamide gel electrophoresis
PAF	platelet-activating factor; (1-o-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylchlorine)
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
pBSK-	pBluescript SK-

PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PF4	platelet factor 4
PG	prostaglandin
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PKC	protein kinase C
PLC, D	phospholipase C, D
PMSF	phenylmethylsulfonyl fluoride
Poly (A) RNA	polyadenylated RNA
r	recombinant
RANTES	Regulated on Activation, T cell Expressed and Secreted
RNA	ribonucleic acid
cRNA	copy RNA
mRNA	messenger RNA
RNase A	ribonuclease A
ROCC	receptor operated calcium channel
RP-HPLC	reverse phase high pressure liquid chromatography
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of mean
sLe^x	sialyl $Lewis^x$
SSC	standard saline citrate
Taq polymerase	*Thermus aquaticus* polymerase
TBE	tris borate EDTA buffer
TBS	tris-buffered saline
TEMED	tetramethylethylenediamine
TFA	trifluroacetic acid
$TGF\alpha/\beta_1$	Transforming growth factor alpha/ $beta_1$
Th cell	T helper cell
TNF	tumour necrosis factor
7-TM receptor	7-transmembrane receptor
Triton-X	octylphenoxyolyethoxyethanol
Tris	tris (hydroxymethyl) aminomethane
TSPS	thrombin-stimulated platelet supernatant
Tween 20	polyoxyethylene-sorbitan monolaurate
TX	thromboxane
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late activation antigen-4
WEB 2086	(3-[4-(2-chlorphenyl)-9-methyl-6H-thieno [3,2-f] [1,-2,4]-triazolo-[4,3,a] [1,4]-diazepin-2yl]-1-(4-morpholinyl)-propanone
ZAP	zymosan-activated plasma

CHAPTER 1. INTRODUCTION

1.1 Leukocyte migration is a primary host defence mechanism

Leukocyte migration is a primary host defence mechanism allowing the infiltration of phagocytes into inflammatory sites and development of an immune response. The Roman writer, Cornelius Celsus, defined the four 'cardinal signs' of inflammation as redness and swelling with pain and heat. The significance and explanation for these observations was not realised until the beginning of the 19th century in the early pioneering work of Cohnheim, Mechnikoff and Erlich (reviewed in Ryan & Majno, 1997). By irritating the mesentery and skin from the tongue of frogs, Julius Cohnheim (1882) observed a series of vascular changes analagous to Celsus' signs. Initially he noted the dilatation of blood vessels and that the blood flow increased first in the arteries, then the veins, and finally the capillaries. The blood flow slowed especially in the veins (venules) and leukocytes were noted to accumulate and adhere as a layer along the vessel wall before moving out of the vessel. Some years later, he suggested that the reduction of flow, sluggish movement of cells and concurrent swelling of the tissue might be due to plasma release into the surrounding tissue. The reason for white blood cell movement into the extravascular tissue was not fully appreciated until Metchnikoff (1893) suggested that these cells were capable of phagocytosing particles including bacteria, and in this way the offending irritant could be removed .

The conclusions of Cohnheim and Mechnikoff were surprisingly prophetic and form the basis of current thinking on leukocyte migration. Springer and others (1990) have suggested a scenario of precise co-ordinated events which mediate the extravasation of cells. Initially, inflammatory mediators cause leukocytes to leave the main vascular flow and marginate (roll) along the surface wall; an action which requires attachment to

and detachment from the endothelium. This phenomenon is achieved by rapid binding kinetics (tethering) of sialyl-Lewis^x (sLe^x) on the leukocyte surface to selectins such as E-selectin (Bevilacqua *et al.* 1987), on the endothelium. This process is low affinity and is replaced by high affinity leukocyte adherence between β_2-integrins expressed on the leukocytes and members of the Ig superfamily - intercellular adhesion molecule (ICAM; Rothlein *et al.* 1986; Dustin *et al.* 1986) and vascular cell adhesion molecule (VCAM; Elices *et al.* 1990), on the endothelial surface. Firm adhesion is followed by transmigration through endothelial cell junctions and migration along a chemotactic gradient to the site of the stimulus for inflammation.

Mechnikoff's discovery of phagocyosis explained the reason for the existence of such a cascade; to allow pathogens to be rapidly cleared from the tissue. The processes of phagocytosis, presentation of antigen with major histocompatability complex to T cells and subsequent assistance of antibody production by B cells, are well documented (Male *et al.* 1989). The mechanisms by which the necessary cells are selectively recruited to a site of inflammation appears to involve complex networks of inflammatory mediators. The dissection of the role of individual mediators in various inflammatory conditions has been the source of much research over the past few decades not least in the search to identify novel therapeutic targets.

1.2. Asthma: an inflammatory disease

The first description of clinical asthma was by Hippocrates in 4 B.C. detailing a condition of breathlessness and deriving the name from the Greek work meaning 'panting'. However, descriptions of a seasonal disorder which was treated with an extract of the plant *Ma huang*, a source of the catecholamine ephedrine, are documented in Chinese medicine dating between 2500-1000 B.C. (reviewed by Gould & Raffin, 1995). The association between asthma and inflammation was not recognised until around the turn of this century. Virchow documented that patients at status asthmaticus had airways obstructed by mucus plugs which contained eosinophils. At around the same time, Osler, in 'The Principles and Practice of Medicine,' 1892 (reviewed by Frew 1996) reasoned that the cause of such obstruction might be due to excessive spasm of the bronchial smooth muscle, inflammation of the airways or caused by abnormalities in neural control. It was a long held belief that bronchospasm was the basis of the underlying pathophysiology giving rise to the reversible limitation of airflow (reviewed in Keeney, 1997). Observations that some degree of bronchial inflammation was present in all asthmatics were not reported until the 1960s (Glynn et al 1960, Salvato 1968) and verified by more extensive studies during the early 1980s with the advent of fibre-optic bronchoscopy. Of note, studies by Laitinen *et al.* (1985) demonstrated the presence of epithelial destruction and exposure of superficial intraepithelial nerves, even in mild asthmatics. The authors suggested that these nerves may be unprotected from specific and non-specific stimuli, thereby linking airways inflammation with hyperreactivity. It is now recognised that the underlying pathology of asthma is airway inflammation, and that bronchodilatory therapy offers only symptomatic, albeit rapid, relief.

1.3. Clinical definition of asthma

A definition of asthma as proposed by the American Thoracic Society (American Thoracic Society, 1987) is widely accepted as providing a guideline of the most common and stereotypical features of the disease.

'Asthma is a clinical syndrome characterised by the increased responsiveness of the tracheo-bronchial tree to a variety of stimuli. The major symptoms of asthma are paroxysms of dyspnea (breathlessness), wheezing and cough, which may vary from mild and almost undetectable to severe and unremitting (*status asthmaticus*). The primary physiological manifestation of this hyperresponsiveness is variable airways obstruction. This can take the form of spontaneous fluctuations in the severity of obstruction, substantial improvements in the severity of obstruction following bronchodilators or corticosteriods, or increased obstruction caused by drugs or other stimuli...'

The airway obstruction associated with asthma is largely reversible and clinical monitoring of the severity of the disease commonly involves assessment of changes in the forced expiratory volume in one second (FEV_1) in response to a direct acting bronchoconstrictor, for example metacholine or histamine (Bucca *et al.* 1993). However, enhanced responsiveness is a feature other lung diseases, such as chronic bronchiolitis (Pride, 1993), and even though the reversibility of constrictor responses may be less applicable in such cases, the diagnosis of asthma clearly rests on other criteria. Commonly, for example, asthma demonstrates a circadian rhythm with a worsening of airway function at night or in the early morning. Sensitization through the regular exposure to environmental stimuli has been widely implicated as the cause of persistent asthma, and inhalation of that stimulus is the key method of delivery since bronchial asthma is a disease of the upper airways (Pride, 1993).

Characterisation of stimuli allows some division within the asthmatic population. Allergens (usually large proteins) such as house dust mite (especially *Der P* 1), animal dander (small animal, cat, dog), pollens (grass, ragweed) and fungal spores (eg.

Aspergillus spp.) are common precipitating factors for so-called atopic ('extrinsic') asthma describing a propensity to generate high IgE levels. The classic type I hypersensitivity response to injection of the allergen into skin has traditionally provided the clinician with a diagnostic tool. However atopy is present in only two-thirds of all asthmatics and is distinct from the so called 'intrinsic' sufferers who more commonly demonstrate occupational, aspirin, or smoking-associated asthma (Pride, 1993). This division is somewhat simplistic since Burrows *et al* (1989) have demonstrated that IgE levels peak in late childhood and decrease through adult life; age-corrected IgE levels correlate with the presence of asthma *per se* and is a more effective marker of the disease than skin-prick tests. In addition to the aforementioned specific stimuli, a number of non-specific factors exacerbate asthma and include pollutants, exercise, cold/dry air and stress (Pride, 1993).

The morphological analysis of asthmatic lung demonstrates characteristic hallmarks of the disease. Interestingly, samples from both intrinsic and extrinsic asthmatics demonstrate similar pathology and it has been suggested that the lung can only respond in a finite number of ways regardless of stimulus (Bentley *et al.* 1992). The gross features of advanced asthma include over-inflation of the lungs, and a thickening of the bronchi walls as well as mucus plugs in the upper airways that on closer examination contain shed epithelium, eosinophils, eosinophil-derived Charcot-Leyden crystals and cell debris. Histologically, the mucosa shows signs of oedema formation and infiltration of large numbers of eosinophils and to a lesser extent monocytes and T lymphocytes but not neutrophils (Dunnill 1960; Jeffery 1993). Furthermore there is evidence of lung remodelling; hypertrophy of the bronchial smooth muscle, enlargement of mucous glands, denudation of areas of the respiratory epithelial and thickening of the remaining reticular basement membrane (Laitinen *et al.* 1985, Jeffery 1993).

1.4. Inflammatory response in asthma and models of allergic inflammation

Although naturally occurring asthma exists in very few species, allergic pulmonary inflammation can be induced in a number of species using a sensitisation regime to antigen (commonly ovalbumin, OA) which generates high levels of immunoglobulin and hyperresponsiveness to bronchoconstrictors. However, the guinea pig has been the species of choice for many investigators because of an ability to closely mimic a number of the features of human asthma, including an allergen-induced early and late phase fall in lung function, bronchial hyperresponsiveness (BHR, Hutson *et al.* 1988), as well as an eosinophilic and T lymphocyte-rich infiltrate into the lungs (Dunn *et al.* 1987; Frew *et al.* 1990).

Much work has been undertaken to elucidate the cellular mechanisms of the development of asthma and allergic inflammation using antigen challenge studies in sensitised atopic individuals and in a variety of animal models. Two distinct phases are observed. The immediate phase is characteristically mast cell-driven and the release of preformed mediators results in bronchoconstriction within 10-20 minutes, resolving within 1-2 hours (Holgate *et al.* 1985). Such a mechanism would explain the speed of onset of an asthma attack. By definition, this phase is established by sensitisation (generation of specific IgE, or in the case of the guinea pig IgG_1) following prior exposure to an allergen. Subsequent exposure results in the binding of that allergen to the IgE on the mast cell surface, the triggering of high affinity receptors FcεRI (Metzger *et al.* 1986), mast cell membrane depolarisation and ultimately the release of biologically active substances (Plaut *et al.* 1989). This phase is considered a Type I hypersensitivity response and characterised by mucosal reddening, small vessel

dilatation and oedema; features akin to those of the classical reddening, wheal and flare of Lewis's triple response in skin (Lewis, 1927).

A number of subjects experience a second prolonged fall in lung function the onset of which occurs at 6-12 hours and resolves by 24-48 hours (O'Bryne *et al.* 1987). This is characterised by a marked eosinophil (De Monchy *et al.* 1985) and T lymphocyte (Azzawi *et al.* 1990) infiltration into the lung and bronchial lumen, the hallmarks of the asthmatic lung. Asthma is clearly a chronic cell mediated immune response with aspects of both phases following each repeated exposure to allergen. However, with increasing severity of disease the situation is probably complicated by remodelling of lung tissue (Stewart *et al.* 1993).

The plethora of evidence from such models points to the development of asthma through a complex network of communication between many cell types. The circumstantial evidence for cell and mediator involvement is discussed in the following sections.

1.5. Cells involved in allergic inflammation

1.5.1. The mast cell and basophil

The mucosal surface of the bronchus harbours the tissue fixed mast cell which has traditionally be seen as pivotal in the development of an immediate allergic response (Beasley *et al.* 1989). Mast cells are differentiated from extravasated $CD34^+$ precursors under the influence of c-kit ligand also known as stem cell factor and CD117 (Valent, 1994). The predominant mature mast cells in the human lung (M_T) contain tryptase but not chymotrypsin, characteristic of those which predominate the skin and submucosae. Following initial exposure to an antigen, that antigen is processed by antigen presenting cells (APCs) and in conjunction with major histocompatability complex (MHC) is presentated to T cells. The T helper cell stimulates B cell production of specific IgE (Male *et al* 1989) which binds to the mast cell surface via the high affinity receptor, FcεRI (Metzger *et al.* 1986). On subsequent exposure, cross-linkage of receptor-bound IgE leads to tyrosine kinase phosphorylation; a key event in mast cell degranulation (Paterson *et al.* 1976; Plaut *et al.* 1989). A range of inflammatory mediators are released from mast cell granules or synthesised *de novo* from membrane lipids as summarised in diagram 1.1. The repercussion of degranulation is thus a rapid bronchoconstriction, an upregulation in the production of mucus and increases in vascular permeability, which is aided by the close proximity of mast cells to blood vessels (Eady *et al.* 1979). Furthermore, the release of chemotacic and haemopoietic factors encourages additional cell migration and differentiation (Plaut *et al.* 1989; Bradding *et al.* 1994; Wodnar-Filipowicz *et al.* 1989). Accordingly, increased BAL levels of histamine measured in allergic asthmatics are associated with BHR (Casale *et al.* 1987). Furthermore, recent work by Lukacs and colleagues (1996b) has shown that c-kit ligand influences both histamine levels and eosinophilia in a murine model of

allergic inflammation and c-kit ligand may stimulate and induce the release of some mast cell products directly (Valent, 1994).

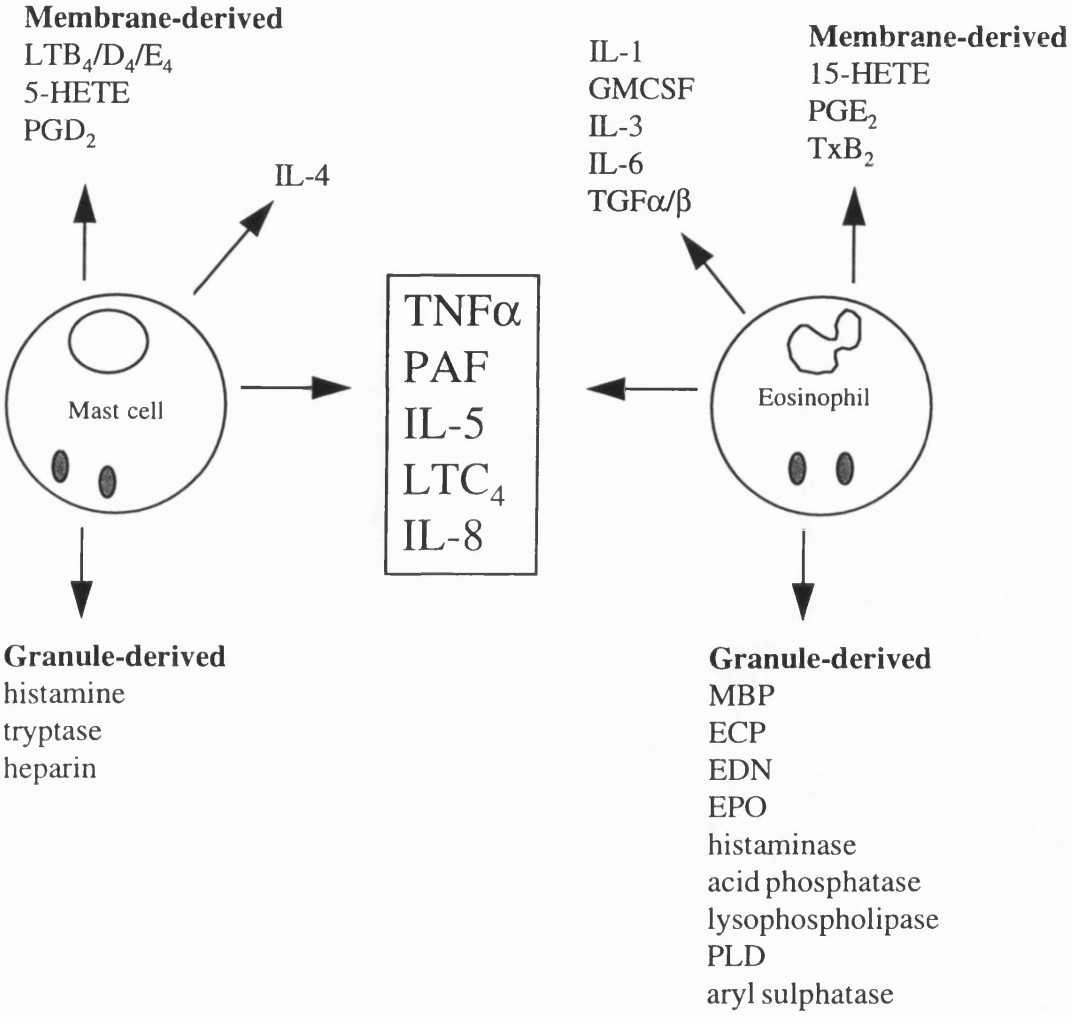


Diagram 1.1 Mast cell and eosinophil products. Central box represents mediators common to both cell types.

Heparin and related proteoglycans are also mast cell products and are able to bind histamine, cationic proteins from the eosinophil as well as the basic chemokines (Witt *et al.* 1994) by virtue of their anionic nature (Page 1991). Furthermore, heparin diminishes the immediate response to antigen in the skin and lung of allergic human subjects (Bowler *et al.* 1993). In addition, studies by Teixera *et al.* (1993) report that heparin suppresses eosinophil accumulation but not oedema formation, indicating that it may have inhibitory properties. These observations have lead to the suggestion that the mast cell may produce natural anti-inflammatory reagents (Page, 1991). However, binding to heparin does not appear to neutralise chemokines (J. Westwick, personnal communication) and in fact may 'present' them in such a way that alters their biological effect on a target cell (Webb *et al* 1993).

Histological examination of the late phase pulmonary response suggests that the predominant histamine-releasing cell is the basophil (Guo *et al.* 1994). They are morphologically distinct from the mast cell in containing fewer cytoplasmic granules and a polylobed nucleus (Wheater & Burkitt, 1987). These cells develop from different precusors than the mast cell, which do not express c-kit and differentiate under the influence of IL-3 which is enhanced by IL-4. Thus, release of IL-3 and IL-4 from mast cells (Plaut *et al.* 1989) encourages progression of the inflammatory reaction towards the late phase and the subsequent down regulation of c-kit ligand by IL-4 may act to modulate histamine release (Valent, 1994).

Both the mast cell and basophil can be activated by both IgE-dependent and independent mechanisms. Mediators that directly induce histamine release from basophils have recently been identified as the monocyte chemotactic proteins, macrophage inflammatory protein -1α and RANTES; members of the CC chemokine family (Kuna

et al. 1993; Alam *et al.* 1992b; Alam *et al.* 1994a), all of which are associated with the late phase of an allergic response.

1.5.2. Eosinophil

1.5.2.1. Life cycle

Ehrlich (1879, see Weller for review) first described the eosinophil as a bilobed leukocyte identified by the presence of specific granules in the cytoplasm that avidly bind to negatively charged dyes including the brominated fluorescein compound, eosin. They account for only 1-6 % of leukocytes in the circulating blood - $1.3\text{-}3.5 \times 10^9$ in the healthy human subject, although as many as 15 times that number may be present as precursors in bone marrow and 100 times as mature cells in tissues.

Eosinophils share the same bone marrow progenitor as neutrophils and basophils (myeloblast) before sequential differentiation into committed eosinophil myelocyte, metamyelocyte and mature eosinophil (Fischkoff *et al.* 1984). Differentiation is mediated by IL-3, IL-5 and GMCSF (Ingley & Young, 1991; Lopez *et al.* 1987, Campbell *et al* 1987), cytokines which are also able to prolong the survival of the mature cell (Rothenberg *et al.* 1988; Yamaguchi *et al.* 1988; Lopez *et al.* 1986). Of these three cytokines, only IL-5 is a specific activator of eosinophil function (Lopez *et al.* 1988). Maturation of the eosinophil within the bone marrow progresses over 2-6 days. The lifespan thereafter is not clear; some authors suggest a half life in the blood of between 6-12 hours (Kay 1985), although in culture with the above cytokines, the eosinophil can be maintained for at least 14 days (Rothenberg *et al.* 1988). Furthermore following extravasation via the post-capillary venules, localisation within the mucosae, where they are particularly abundant appears to maintain survival for weeks (Kay 1985).

The selective recruitment of the eosinophil from the blood into tissue is widely accepted to be at least partly mediated by the ($\alpha_4\beta_1$) integrin VLA-4 (CD49d/CD29), expressed constitutively on eosinophils but not neutrophils (Walsh *et al.* 1991; Weller *et al.* 1991). Adhesion to the endothelium is via VCAM-1 which can be upregulated by TNFα and IL-1 (Dobrina *et al.* 1991). IL-4, another cytokine implicated in the pathogenesis of asthma, appears to upregulate VCAM-1 selectively, but not E-selectin or ICAM-1 (Schleimer *et al.* 1992). The importance of VLA-4 interactions has been verified *in vivo*. Weg *et al* (1993) demonstrated that pre-treatment of ^{111}In-labelled guinea pig eosinophils with the monoclonal antibody HP1/2 (directed against the α_4 subunit of VLA-4) prevented their accumulation into skin sites. Since the number of circulating ^{111}In eosinophils remained constant, it is unlikely that the inhibition was caused by mAb-induced eosinopaenia.

Whilst VLA-4/VCAM-1 provides a specific mechanism through which eosinophils but not neutrophils might be recruited, many investigators have suggested ICAM-1 or E-selectin also mediate the adhesion of eosinophils (Kyan-Aung *et al.* 1991; Kuijpers *et al.* 1993). However their ligands, CD11/CD18 and sLe^x respectively, are also present on a range of leukocytes including neutrophils. ICAM and E-selectin are expressed constitutively on the endothelium although can be upregulated by IL-1, TNFα and IFN-γ (Pober *et al.* 1986). These authors reported that this upregulation varies with time; E-selectin peaked at 4 hours, ICAM-1 at 24 hours, and VCAM-1 at around 12 hours suggesting that such a mechanism might regulate leukocyte infiltration into an inflammatory site. Studies by Wegner and co-workers (Wegner *et al.* 1990; Gundel *et al.* 1991b), using chronic and acute primate models of pulmonary inflammation suggested that distinct time-courses of adhesion molecule expression are relevant *in*

vivo. Repeated exposure to antigen increased ICAM expression and the associated eosinophilia and BHR could be reduced by pre-challenge treatment with anti-ICAM-1 mAb (Wegner *et al.* 1990). However, a single exposure to antigen increased E-selectin but not ICAM-1 expression and administration of anti-E-selectin mAb decreased the observed neutrophil accumulation (Gundel *et al.* 1991b).

1.5.2.2. Activation of eosinophils

Human eosinophil activation through both IgE and and IgE-independent mechanisms is mediated through a number of surface receptors outlined below. The eosinophil is a producer of, as well as target for, many of the mediators (see diagram 1.1), highlighting the possibility for self-perpetuating feedback mechanisms.

MEDIATOR	RECEPTOR	RESPONSE	REFERENCE
LTB_4	Identified on guinea pig eosinophils	Chemoattractant	Sehmi (1992a)
PAF	Evidence of two PAF receptors on eosinophils Kroegel (1989) Graf *et al.* (1983)	Chemoattractant Degranulation LTC_4 generation	Wardlaw (1986) Kroegel (1988, 1989)
IL-3 ,IL-5, GMCSF	High affinity receptors with common β-chain and cytokine specific α chain	Priming effects Induce hypodensity	Sehmi (1992b), Rothenberg (1988)
C5a	C5aR	Potent Chemoattractant	Gerard (1989)
C3a	Likely, but not yet identified	Chemoattractant	Daffern (1995)
RANTES/MIP-1α	CCR1	Chemotaxis, Ca^{2+} mobilisation	Proudfoot (1995)
Eotaxin/MCP3/ RANTES	CCR3	Chemotaxis, Ca^{2+} mobilisation	Daugherty (1996)
IgE	FcεRII; low affinity	Degranulation	Grangette (1989)
IgG	FcγRII	Degranulation	Kaneko (1995)
IgA/secretory IgA	FcαR	Degranulation	Abu-Ghazaleh (1989)

Table 1.1. Summary of receptors on the surface of the human eosinophil

1.5.2.3. Eosinophil activity following stimulation

Increases in $[Ca^{2+}]_i$

Increases in $[Ca^{2+}]_i$ can trigger a number of events in a leukocyte including cellular proliferation, degranulation and changes in gene expression. It is a valuable model of cell activation that can be easily monitored by use of Ca^{2+}-binding fluorescent dyes such as fura2 (Grynkiewicz 1985). In many non-excitable cells, elevation of $[Ca^{2+}]_i$ is essentially achieved by either release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum or influx via transport across the lipid bilayer using receptor operated calcium entry. Ca^{2+} release from intracellular stores is widely accepted to be triggered by Ins(1,4,5)P_3 (IP_3) interaction with IP_3-sensitive Ca^{2+} release channels, which is produced after receptor stimulation by hydrolysis of PIP_2 by PLC. This hydrolysis also generates DAG which initiates the activation of PKC. However, influx is responsible for the sustained phase of elevated $[Ca^{2+}]_i$ and to replenish intracellular stores. Agonist stimulated Ca^{2+} influx can be stimulated by second messengers such as IP_3, phosphatidic acid although the exact nature of this is not known. In addition Ca^{2+} influx may be induced by the emptying of intracellular stores (reviewed in Fasolato, 1994). PAF activates eosinophils using the classical pathway of IP_3-mediated release from Ca^{2+} stores (Kernen *et al.* 1991) and there is evidence that PKC regulates PAF-induced responses (Kroegel *et al.* 1994). In contrast, Grix *et al* (1995) have suggested that increases in $[Ca^{2+}]_i$ in human eosinophils induced by C5a are predominantly due to increased influx across the cell membrane.

Eosinophil chemotaxis

Eosinophils share several properties with most other leukocytes, notably the ability to migrate towards concentration gradients of chemotacic molecules. Clearly *in vivo* this ability is utilised during extravasation, and *in vitro* can be demonstrated using a modified Boyden chamber apparatus (Falk *et al.* 1980). Inducers of eosinophil chemotaxis include PAF (Wardlaw *et al.* 1986), C5a, IL-8 (Kernen *et al.* 1991) and RANTES (Rot *et al.* 1992) through interaction with G protein-coupled seven transmembrane receptors (Kernen *et al.* 1991). However the signal transduction processes that govern cell migration in eosinophils are not well defined. Studies by Schweizer (1994) and Kernen (1991) suggests that shape change and chemotaxis in eosinophils occur independently of the classical signal transduction pathway involving phospholipase C, increases in $[Ca^{2+}]_i$ and protein kinase C activation. In contrast, Elsner *et al* (1996) reported that eosinophil migration could be inhibited by chelation of intracellular calcium. However, cell migration is dependent on the reversible polymerisation of globular monomeric actin into filamentous polymers of actin and is an immediate intracellular response after addition of a chemoattractant (Stossel 1993). In eosinophils, PAF induces maximal actin polymerisation by 10-15 sec and is particularly evident after priming with IL-5 (Schweizer *et al* 1994).

Alterations in eosinophil density

Some stimuli initiate a change in the phenotype and reactivity of eosinophils from an unstimulated 'normodense' to a hypodense state. Hypodense eosinophils show evidence of degranulation through a decrease in the number and size of granules (and hence eosin staining is weaker) and consequently separate at a lower sedimentation density on a discontinuous Percoll gradient (Winqvist *et al.* 1982). They are also more reactive to

stimulants such as PAF (Yakawa *et al.* 1989), exhibit a higher oxygen consumption (Winqvist *et al.* 1982), generate more LTC_4 in response to activation with calcium ionophore (Kajita *et al.* 1985), express increased levels of IgE low affinity receptors (Capron *et al.* 1984) and demonstrate enhanced chemotactic activity (Wardlaw *et al.* 1986). This state would seem to have relevance *in vivo* since many investigators have demonstrated increased numbers of hypodense eosinophils in clinical eosinophilia (Yakawa *et al.* 1989) and in the peripheral blood of asthmatic patients (Frick *et al.* 1989).

Release of cationic proteins

The anti-parasitic ability of the eosinophil is achieved through the release of a number of basic proteins (14,000-21,000 Daltons) from the crystalloid core of the specific granules which are helminthotoxins. Major basic protein (MBP, Hastie 1987; Gleich *et al.* 1979) is the most abundant but is aided by the actions of eosinophil cation protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO), an enzyme which catalyses the formation of hypohalous acids (Weller 1991). However, these proteins are also detrimental to host cells and contribute to the epithelial damage observed in asthma by causing exfoliation, ciliostasis and cytotoxicity (Gleich *et al.* 1979).

1.5.2.4. Eosinophils and bronchial hyperreactivity (BHR)

Eosinophils are a hallmark of asthma and characteristic of the late phase response. There has been much circumstantial evidence supporting a causative role for the eosinophil in the development of BHR. Patients with asthma have increased numbers of eosinophils in the BAL and these cells are associated with the late phase reaction (LPR) following allergen provocation in sensitised atopic subjects (De Monchy *et al.* 1985; Wardlaw *et al.* 1988). Some authors have suggested that the observed increases of sputum eosinophils in asthma might be a suitable prognostic marker for asthmatic exacerbations provided no bronchial infection was present (Ferguson *et al.* 1995). Furthermore, a number of investigators have demonstrated evidence of an inverse correlation between eosinophil counts in peripheral blood and specific airway conductance (FEV_1) in patients with active bronchial asthma (Horn *et al.* 1975; Ferguson *et al.* 1995) but positive correlation to BHR (Durham & Kay, 1985). Such trends have been successfully replicated in the OA-sensitised guinea pig model (Sanjar *et al.* 1990; Frew *et al.* 1990).

Some authors have argued that quantification of eosinophil activation might be a more accurate assessment of the presence of asthma. Increases in eosinophil-derived cationic protein can be detected in the serum (Griffin *et al.* 1991), sputum (Ferguson *et al.* 1995), BAL (Wardlaw *et al.* 1988) and bronchial biopsy samples (Filley *et al.* 1982) from asthmatics. A number of studies correlate levels of ECP with the severity of disease (Bousquet *et al.* 1994; Zimmermann *et al.* 1993). Cationic proteins released from eosinophils are able to damage epithelium (Gleich *et al.* 1979) and this may result in a denudation of areas of the airways epithelium, thus exposing underlying nerves. Through this mechanism, eosinophils could cause hyperresponsiveness and Gundel *et al* (1991a) have demonstrated that human MBP induces BHR and constriction in primates.

Furthermore, an important study by Lefort *et al* (1996) demonstrated that the neutralisation of major basic protein inhibited antigen-induced bronchial hyperreactivity in sensitised guinea pigs. However conflicting evidence links elevated levels of these proteins *in vivo* with the development of BHR. For example, Zimmerman *et al* (1993) detected a correlation between serum ECP levels and FEV_1 in symptomatic asthmatic children but not in asymptomatic subjects, whilst Ferguson *et al* (1995) reported no correlation between lung function (PC_{20} for histamine) and ECP levels for some chronic asthmatics. Furthermore a number of investigators have reported observations of hyperreactivity without inflammation and vice versa leading to the hypothesis that eosinophils and BHR reflect a common origin but are not necessarily causally related (Owaka *et al.* 1981). It is feasible that in some circumstances other cells might drive hyperresponsiveness such as the mast cell or the platelet possibly through PAF, a mediator able to induce the migration of human eosinophils (Wardlaw *et al.* 1986) and BHR in some subjects (Coyle *et al.* 1990).

1.5.3. T lymphocytes

The lymphocyte is pivotal in the recognition of foreign antigen and the T cell assists antibody formation, stimulates cytotoxic effects of other cells such as the macrophage and regulates the level of the immune response (Male 1989). $CD4^+$ T cells are implicated as central to the pathogenesis of asthma by virtue of the production of a panel of cytokines from the so-called T helper 2 lymphocytes which favour recruitment of eosinophils (see reference Mosmann *et al.* 1986 and later sections of this introduction). In addition, direct evidence exists for the presence of T lymphocytes in asthma. Kay and colleagues identified increased numbers of activated ($CD25^+$) T lymphocytes in the bronchial mucosa (Azzawi *et al.* 1990, Hamid *et al.* 1991) and in the blood of

asthmatics (Corrigan *et al.* 1988). Corrigan *et al* (1993) have also demonstrated increased IL-5 levels, a product of Th2 responses in the serum of asthmatics following $CD4^+$ lymphocytes activation. Furthermore T lymphocytes are specifically a feature of the late phase response to allergen in the human lung (Metzger *et al.* 1987) and mRNA for Th2 mediators (IL-5, IL-4, IL-10) is increased following allergen challenge in the BAL cells of atopic subjects (Robinson *et al.* 1992).

1.5.4. The alveolar macrophage

The differentiation of the blood-derived monocyte into the tissue macrophage (Bitterman *et al.* 1984) produces a cell with highly effective phagocytic (Gieser *et al.* 1994) and tumorocytic properties (Weissler *et al.* 1986), that appears to be able to mediate the immune response through the release of a range of inflammatory mediators.

Following release of monocytes into the blood from the bone marrow, extravasation occurs after 24 hours (Issekutz *et al.* 1993). A number of members of the chemokine family, notably the macrophage inflammatory proteins (Fahey *et al.* 1992), RANTES (Schall *et al.* 1988) and especially the monocyte chemoattractant proteins (Rollins *et al.* 1991) have been implicated in their recruitment into sites of inflammation. Human monocytes undergo differentiation upon migration, although the process occurs spontaneously when monocytes are culture *in vitro* for a few days (Kaplan & Gaudernack, 1982). This process confers a change in the secretory capacity of these cells. The extent of maturation thereafter is thought to be responsible for the large heterogeneity within the macrophage population, which can be observed experimentally by differential fractionation on Percoll gradients and cytokine release (Elias *et al.* 1985; Gessani *et al.* 1993).

The physiological role of the macrophage in the lung is to clear inhaled pathogens (Green & Kass, 1964) and cell debris and it is the most abundant cell in normal BAL fluid. They are likely to play a role in the pathogenesis of asthma since:

1. Alveolar macrophages and blood monocytes express the low affinity IgE receptor, FcεRII, (Capron & Prin, 1986) and retain the ability to respond to antigen (Rankin *et al.* 1982). There is some evidence to suggest that expression is increased in asthmatic subjects (Vecchiarelli *et al.* 1994). Their primary location in the air spaces would allow exposure to antigen and trigger factors.

2. The activated macrophage releases a vast array of proinflammatory mediators which include the leukotrienes (Rankin *et al.* 1982) and PAF which are bronchospasmogens and chemoattractants, the early cytokines TNF (Gessani *et al.* 1993) and IL-1 (Elias *et al.* 1985), the haemopoietic factors GMCSF, IL-3 and IL-5 as well as chemokines IL-8 (Standiford *et al.* 1990b), MIP-1α (VanOtteren *et al.* 1994; Berkman *et al.* 1995) and MCP-1 (Brieland *et al.* 1993). The production of some of these mediators by macrophages may be increased in asthmatic subjects (Damon *et al.* 1987).

1.5.5. Platelet

The platelet is an anucleated cell element originating from fragmentation of megakaryocytes in the bone marrow or possible within the capillary network of the lung. It normally circulates as a single entity in the peripheral blood but can adhere to exposed subendothelial matrix proteins using a number of surface membrane integrins (Herd & Page 1994). Furthermore, platelets express P-selectin through which they may bind leukocytes (Springer 1990).

Activation of the platelet causes the release of a number of mediators, reviewed in reference Herd & Page 1994, which are known to induce bronchoconstriction (PAF, adenosine, TXA_2), smooth muscle and fibroblast proliferation associated with lung remodelling (PDGF, βTGF), vasoconstriction and dilatation (5-HT, PGE_2) as well as airway hyperresponsiveness (PAF) and eosinophil chemotaxis (PAF, RANTES). Whilst these are features of the acute and chronic asthmatic response during which a number of the mediators are upregulated, this does not provide unequivocal evidence for the involvement of platelets in asthma as many of these mediators are manufactured by other cell types (Sanchez-Crespo *et al.* 1980). More compelling evidence comes from an expression of low affinity IgE receptors FcεRII (Capron & Prin, 1986) and an ability to migrate following antigen challenge (Zhang *et al.* 1993). Furthermore platelets have been found in the BAL of allergic rabbits and asthmatic human subjects during late onset airways obstruction (Herd & Page, 1994). *In vivo* studies have indicated that the ability of PAF to induce bronchoconstriction (Vargaftig *et al* 1980), bronchial hyperresponsivness and associated eosinophilia (Coyle *et al* 1990) is dependent on the presence of platelets. Some authors (Coyle *et al* 1990) have suggested that this might be largely attributed to the actions of PAF-induced PAF release from platelets; in the guinea pig PAF-acether administration causes eosinophil recruitment in the lung which can be reduced by PAF antagonists (Lellouch-Tubiana *et al.* 1988). However, the human platelet α granules are a rich source of pre-formed chemokines including platelet factor 4, platelet basic protein and derivatives CTAP-III, βTG (Holt *et al* 1986), PF4 (Walz *et al.* 1989) as well as the CC chemokine, RANTES (Kameyoshi *et al.* 1992). It is possible that the latter, which is an eosinophil attractant (Alam *et al.* 1993) and other as yet unidentified platelet-derived chemokines, may also contribute to airways hyperreactivity.

1.6. Factors which mediate allergic inflammation

It is widely accepted that distinct mediators govern the characteristic features of an allergic inflammatory response. Many of these substances have been identified, implicated on the basis of their upregulation, providing a picture of complex cell-mediated interactions. However, there appears to be a high level of functional redundancy. The use of novel therapeutic reagents and monoclonal antibodies has enabled some dissection of the relative importance of a given mediator in the development of allergy and the key lines of evidence for each are outlined below.

1.6.1. Histamine

The release of inflammatory mediators from the mast cell, most notably histamine, is widely accepted as the basis of the immediate allergic response (Holgate *et al.* 1985). Histamine is formed from histidine by the action of histidine decarboxylase and is stored within the mast cell and basophil bound to proteoglycan. Histamine is a potent bronchoconstrictor; inhalation of the mediator induces a response within 2-5 minutes lasting approximately 20 minutes. Asthmatics exhibit hyperresponsiveness towards histamine and this is the basis of PC_{20} measurement to assess airway function (Bucca *et al.* 1993). Intradermal injection of histamine induces a characteristic triple response as described by Lewis (1927), highlighting its ability to cause direct vasodilatation (erythema), increase vascular permeability and an erythematous flare through axon-reflex induced vasodilatation. Early antigen challenge studies in the skin by Katz *et al.* (1942) demonstrated that ragweed (antigen) could induce a similar response in sensitised individuals, pointing to the release of histamine. A number of subsequent studies have demonstrated that type I responses can be blocked by combination of H_1

and H_2 receptor antagonists (Woodward *et al.* 1985) although studies by Weg *et al.* (1991) suggest that oedema can only be effectively blocked with combinations of histamine, PAF and leukotriene antagonists (see below). However, despite the benefits of histamine antagonists in the relief of allergic rhinitis, they are not effective at treating allergic asthma. Given the number of mediators with overlapping functions released during chronic inflammation, it is also possible that the selective inhibition of any one mediator may be compensated for by others.

1.6.2. Neuropeptides

Neuropeptides, which modulate the non-adrenergic non-cholinergic (NANC) system are a family of proteins which share some functional characteristics of histamine. Substance P induces a wheal and flare reaction in skin in the immediate but not late phase response (Umemoto *et al.* 1976) and causes airways obstruction through bronchoconstriction and excessive mucus production (Lundberg *et al.* 1983). However vasoactive intestinal peptide, as well as the non-neuropeptide nitric oxide (Barnes, 1995), mediates NANC-bronchodilatory responses, suggesting that an alteration in the balance of neuropeptides could effect airway function.

Bradykinin has been implicated in allergic inflammation by virtue of increased levels in the upper airways of asthmatics following allergen challenge (Proud *et al* 1983). This kinin is a potent bronchoconstrictor in asthmatic but not normal controls (Fuller *et al.* 1987). Since they themselves are weak constrictors of isolated airway smooth muscle, it is possible that their influence is exerted by neural reflexes. A number of studies have demonstrated that administration of bradykinin receptor antagonists prior to antigen challenge in experimental animals inhibits airway hyperresponsiveness (Farmer *et al* 1992).

1.6.3. Complement

Complement is an integral part of the innate immune system and consists of a number of plasma-derived protein mediators with pro-inflammatory activities generated through an amplified cascade triggered by antigen-antibody complexes. The 'anaphylatoxin' C5a can increase vascular permeability by neutrophil-dependent (Wedmore & Williams, 1981a) and independent mechanisms, the latter through stimulating the release of histamine from mast cells (Gerard & Hugli, 1981; Jose *et al.* 1981). Histamine-releasing activity is abolished by the removal of the carboxy terminal arginine (C5a-des Arg), whilst the ability to modulate vascular permeability remains unaffected (Jose *et al.* 1981). Complement can be generated by pathways other than immune complex deposition, which is not a feature of type 1 reactions, however little evidence exists for the involvement of C5a in asthma. However, it is commonly used as a control in an experimental setting by virtue of the potent ability to chemoattract most leukocyte types (Okawa *et al.* 1981).

1.6.4. Eicosanoids

Eicosanoids are a collection of 20-carbon fatty acids metabolised *de novo* from the membrane derived phospholipid arachidonic acid (AA). Further metabolism of free AA generates leukotrienes (LTs) through the action of 5-lipoxygenase and collectively LTA_4-E_4 mediate a wide range of responses. The leukotrienes are potent bronchoconstrictors and are at least a thousand times more effective in this role than histamine (Dahlen *et al.* 1980). Feldberg and Kellaway first observed the actions of these mediators in 1938 after injecting snake venom into perfused lungs which caused a slow, long lasting contraction of the smooth muscle (described in Piper 1993). Later

studies by Brocklehurst (1960) demonstrated that these 'slow reacting substances' (SRSs) were also produced during antigen-challenge in the lung. SRSs were structurally identified by Samuelsson *et al.* (1979) as derivatives of AA and specifically LTD_4 as the major slow-reacting substance of anaphylaxis in the guinea pig (Morris 1980).

LTC_4 and LTD_4 cause direct bronchoconstriction in anaesthetised guinea pigs when given in an aerosolised form (Brocklehurst, 1960) and a prolonged response when given i.v. (Piper & Samhoun, 1981) through the release of TXA_2, which has been shown to contract isolated trachea and increase pulmonary resistance (Piper & Samhoun, 1982). Such long duration of action is akin to the sustained constriction seen in clinical asthma although the involvement of TXA_2 is questionable since indomethacin does not inhibit LT-induced prolonged contractions of the human bronchus (Brocklehurst, 1960). In addition, LTs might also mediate airways obstruction by the induction of mucus hypersecretion (Marom *et al.* 1982).

The leukotrienes are potent at inducing changes in vascular permeability and injection of LTC_4 and LTD_4 induces a wheal and flare response in human skin (Bisgaard *et al* 1982). LTC_4 and D_4-induced changes in vascular permeability are not dependent on the presence of neutrophils (Wedmore & Williams, 1981a), unlike those of LTB_4 which are abolished in neutropenic animals. LTB_4 has previously been shown to be a potent neutrophil (Ford-Hutchinson *et al.* 1980) and eosinophil (Sehmi *et al.* 1991) chemoattractant, and there is some evidence to implicate LTD_4 (Spada *et al.* 1994) and LTE_4 (Laitinen *et al.* 1993) as mediators of eosinophil accumulation. The importance of LTs in the development of allergic inflammation is underlined by increased levels in plasma (Zakrzewski *et al.* 1985) amd BAL (Wenzel *et al.* 1990) following allergen

challenge. Furthermore, a number of LTD_4 antagonists have improved lung function in asthmatic subjects (Spector *et al.* 1994).

In contrast to the predominant effects of LTs inducing changes in vascular permeability, prostaglandins which are derivatives of cyclooxygenase metabolism of AA, markedly increase blood flow (Williams & Peck, 1977). In this role they potentiate the oedematus response and associated pain of a number of other mediators (Ferreira 1972; Williams & Morley, 1973). Such a mechanism explains the paradox of high level of PGs in inflammatory exudates observed by Willis and yet an inability of exogenous PGs to induce oedema themselves. Accordingly, cyclo-oxygenase inhibitors decrease oedema and pain in acute inflammation (Williams & Morley, 1973). However, these inhibitors are of limited use in chronic inflammatory models; indomethacin does not reduce swelling despite a reduction in PG levels (O'Bryne *et al.* 1987).

1.6.5. PAF

The phospholipid, platelet activating factor (PAF) was originally identified from the supernatant of basophils from sensitised rabbits (Benveniste *et al.* 1972) and has been detected at increased levels in the blood (Nakamura *et al.* 1987) and BAL (Count *et al.* 1987) of asthmatics. PAF has been shown to increase vascular permeability in human, rabbit and guinea pig skin (Archer *et al.* 1984; Wedmore & Williams, 1981b; Morley *et al.* 1983) which Weg *et al* (1991) demonstrated could be partially inhibited with the PAF antagonist WEB 2086, *in vivo*. PAF is also able to induce bronchospasm in experimental animals but only in the presence of platelets implying the involvement of other platelet-derived mediators (Vargaftig *et al.* 1980). In addition, PAF is a potent chemoattractant of eosinophils *in vitro* in comparison to eosinophil chemotactic factor

of anaphylaxis tetrapeptides, histamine and LTB_4 (Wardlaw *et al.* 1986). However, Faccioli *et al* (1991) reported increased potency for LTB_4 over PAF to induce ^{111}In-eosinophil migration into guinea pig skin sites, although C5a-des-Arg was considerably more effective than either mediator. Studies by Little *et al* (1991) suggest that responsiveness to PAF may depend on the activation state of eosinophils since hypodense cells were more reactive. However as for many of these mediators, PAF is non selective, also attracting neutrophils and monocytes (Czarnetzki, 1983).

1.6.6. Early-response cytokines

The accumulation of cells at sites of inflammation has been largely attributed to a complex cascade of mediators of which tumour necrosis factor-alpha (TNFα) and IL-1 have been identified as proximal cytokines. Both are produced from almost every nucleated cell suggesting the potential for ubiquitous release, although the monocyte/macrophage is a particularly rich source (Le & Vilcek, 1987). These cytokines have somewhat overlapping actions such as the ability to upregulate the production of a number of inflammatory mediators from target cells. These included most cytokines (notably IL-1 and TNF themselves), chemokines, lipid mediators such as PAF as well as prostaglandins through the stimulation of AA release and increase PLA_2 activity (Pober *et al.* 1986). Studies by Watson *et al* (1988) highlight a role for IL-1 in increasing vascular permeability leading to oedema.

Both TNFα and IL-1 have been widely implicated in the development of allergic inflammation. They are released from antigen-stimulated mast cells (Gordon & Galli, 1990) and Th2 cells (Mosmann *et al.* 1986) and therefore a number of target cells thereafter are activated, consistent with their role as inducers of the inflammatory

cascade. Furthermore, elevated levels of TNFα and another early cytokine, IL-6 have been reported from IgE-stimulated macrophages and BAL leukocytes of allergic asthmatics (Gosset *et al.* 1991; Cembryznska-Nowak *et al.* 1993). Similarly, local generation of IL-1 was detected following antigen-challenge in atopic patients (Bochner *et al.* 1990).

Using *in vivo* animal models, a number of workers have reported that inhibition of IL-1 or TNF reduces leukocyte accumulation. Watson *et al.* (1993) demonstrated a protective effect in ovalbumin sensitised guinea pigs against bronchial hyperreactivity and the associated eosinophilia using the selective IL-1 receptor antagonist (IL-1ra). The authors also noted reduced TNF bioactivity in the BAL. In the mouse, anti-TNF antibodies reduce leukocyte accumulation following IgE mediated in a model of passive cutaneous anaphylaxis (Wershil *et al.* 1991). Since these cytokines do not chemoattract leukocytes directly (Lukacs *et al.* 1995b), leukocyte recruitment *in vivo* may be associated with the TNF/IL-1-induced release of intermediary cytokines and chemokines (Pober *et al.* 1986), VCAM-1 (Dobrina *et al.* 1991) and ICAM-1 (Pober *et al.* 1986) expression on the endothelial cell surface.

1.6.7. Establishment of Th1/Th2 phenotypes

Activated T cells appear to be a characteristic of atopic asthma (Azzawi *et al.* 1990). The identification of distinct murine $CD4^+$ T helper clones by Mosmann *et al* (1986) divided on the basis of selective cytokine secretions aided the understanding of the role of T cells in allergy. Similar specific clones have since been identified in the human (Wierenga *et al.* 1991). Termed Th1 and Th2 phenotypes, Th1 cells predominantly secrete IL-2 and IFNγ whilst Th2 cells produce IL-4 and IL-5 (Mosmann *et al.* 1986) as

well as IL-10 and IL-13 (Minty *et al.* 1993). IL-3 and GMCSF are secreted by both types. In mice it appears that T helper precursors pass through an intermediary Th0 stage secreting IL-2, IL-4 and IFNγ (Mosmann *et al.* 1986). Although IL-2 induces the development of all T cells (Mills *et al.* 1985, Cantrell *et al* 1984), some actions of Th1/Th2 cytokines are broadly antagonistic of one another, for example IL-4 induces IgE production from B cells which is inhibited by IFNγ (Del Prete *et al.* 1988). It is possible that a shift in balance between these phenotypes could be causative of T cell-mediated diseases. Some of the key interactions are summarised in diagram 1.2.

Several lines of evidence point to a role for the Th2 phenotype in asthma. Robinson *et al* (1992) observed T cell mRNA expression for predominantly Th2-like cytokines in the BAL of atopic patients following allergen challenge which was consistent with an increase in activated eosinophils. The secreted panel of Th2 mediators, namely IL-5, IL-4 as well as IL-10, play key roles in allergy. IL-5 (as well as IL-3 and GMCSF) promotes the terminal differentiation of committed eosinophil precursors (Campbell *et al* 1987), selectively activates mature eosinophils (Lopez *et al.* 1988) and prolongs their survival in culture (Yamaguchi *et al.* 1988). Whilst IL-5 is a weak chemoattractant of eosinophils (Wang *et al.* 1989), an ability to 'prime' these cells augments the migratory responses towards potent but non-specific mediators such as PAF (Schweizer *et al.* 1994). This could provide a selective mechanism for eosinophil recruitment. Interestingly, Sehmi *et al* (1992b) observed that PAF-induced eosinophil responses using cells from asthmatic patients could not be augmented by IL-5. The authors suggested that this maybe due to prior exposure *in vivo* possibly due to increased levels of this cytokine. In support of this, a number of studies have demonstrated an upregulation of IL-5 in allergic inflammation. Hamid *et al* (1991) reported a correlation between IL-5 mRNA levels in human bronchial biopsies and $EG2^+$ eosinophils and

$CD25^+$ lymphocytes; markers used to assess severity of asthma. Van Oosterhout (1993) demonstrated that pretreatment using anti-IL-5 antibodies reduced BHR and eosinophil accumulation in the guinea pig lung following antigen challenge. This might be explained by the identification of IL-5 as the predominant eosinophil-active cytokine during the LPR following segmental antigen challenge, at least in the human lung (Ohnishi *et al.* 1993).

Mast cells are also a source of IL-5, released during degranulation following IgE crosslinking by antigen (Plaut *et al.* 1989). The growth and regulation of mast cells and synthesis of IgE from B cells through Ig isotype switching is driven by IL-4, another Th2 cytokine (Finkelman *et al.* 1990). From studies using IL-4 knock-out mice, Kopf and colleagues (1993) reported that IL-4 favoured the conversion of naïve $CD4^+$ T cells to the Th2 phenotype both *in vitro* and *in vivo* and was essential for $CD4^+$-derived IL-5 and the IL-5-induced eosinophilia in this model. IL-4 also modulates other aspects of eosinophil activity, specifically extravasation through the upregulation of adhesion molecule VCAM-1 on the endothelial surface (Schleimer *et al.* 1992). IL-4 (as well as IL-5) appears to be upregulated in allergic asthmatics as assessed by increased expression in $CD2^+$ T cells and protein levels in BAL (Robinson *et al.* 1992; Walker *et al.* 1992). However, recent work by Krug and colleagues (1996) indicated that most T cells from the BAL of asthmatics produce IFNγ when stimulated *ex vivo* whilst those secreting IL-4 accounted for less than 2 %. This work suggests a selective increase in IFNγ production during asthma and underlines the fact that the application of the Th1/Th2 division to human inflammation should perhaps be approached with some caution.

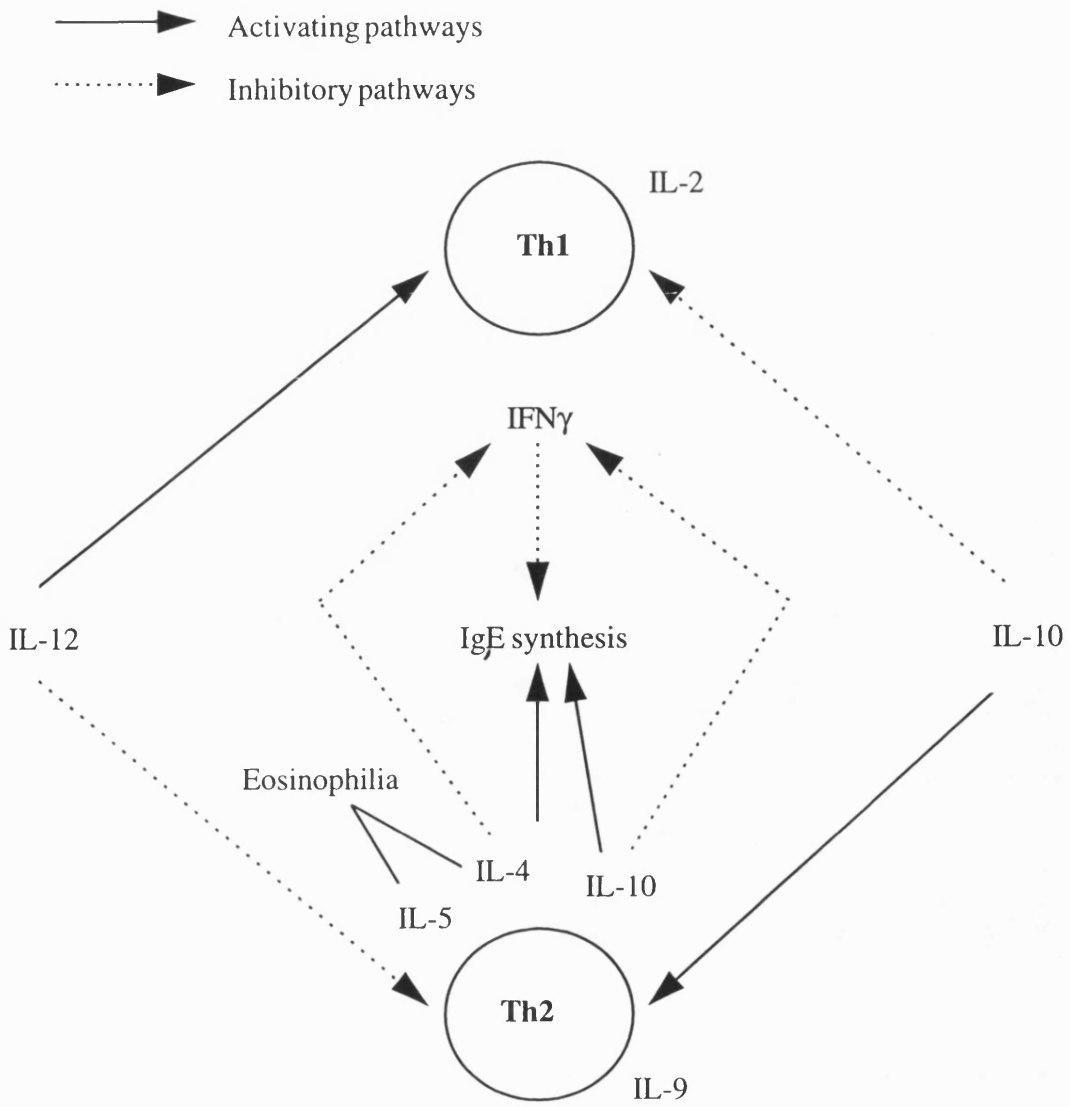


Diagram 1.2 Key Th1/Th2 derived cytokines and functional interplay

1.6.8. Chemokines

1.6.8.1. Overview

The chemotactic cytokines or 'chemokines' are a family of 8-10 kD, basic proteins, each of which is able to attract distinct subsets of leukocytes (Lindley *et al.* 1993a). For this reason, they are widely proposed to be responsible for the selective leukocyte recruitment of inflammation. The chemokines were originally separated into two main families on the basis of structure and function. The division is made on the position of the first two of four cysteine residues being either adjacent (CC) or juxtaposed with another amino acid (CXC, Lindley *et al.* 1993b). However, it would seem that other subfamilies also exist; lymphotactin contains only one N terminal cysteine and probably represents a member of the so-called 'C' family, of chemokines (Kennedy *et al.* 1995), whilst the recent characterisation of 'fractalkine' by Bazan (1997) suggests that chemokines can bear a CX_3C motif. At the time of writing, 11 human CXC chemokines and 9 human CC chemokines had been characterised. Whilst some were identified as biological activity secreted by cells, in tissues or biological fluids (e.g. IL-8, eotaxin), many others were discovered by the cloning of genes expressed in selected cell types (e.g. RANTES).

CXC chemokines such as IL-8 and MGSA, are broadly chemotactic for neutrophils and T lymphocytes, whereas CC chemokines attract monocytes, T lymphocytes, eosinophils and activate basophils (Lindley et al 1993b). Accordingly, the genes for each family are located on separate chromosomes; CXC on q12-21 of human chromosome 4 (Richmond *et al.* 1988) and CC on the q11-21 region of 17 (Irving *et al.* 1990) where the individual loci for each CC chemokine have been designated *Syca* 1-9 or small incucible cytokine a

(reviewed in Youn *et al.* 1995). It has been suggested that the two families diverged many millions of years ago from a single ancestral gene.

Despite the separation into the subfamilies, many features are common to most chemokines:-

Functionally: all chemokines stimulate the directional migration of selected cell types and many induce other features of cell activation such as calcium mobilisation, enzyme release, respiratory burst and adherence to endothelium.

Structurally: most chemokine cDNAs encode a protein of 92-99 amino acids, which includes a cleavable hydrophobic amino acid sequence (approximately 20 residues) at the N terminus. Furthermore the chemokines show striking conservation of exon/intron gene organisation. CXC chemokines assume a 4 exon/3 intron configuration whilst CCs are arranged as 3 exons/2 introns, with the first exon encoding most of the leader sequence and the second and third, the mature protein (Schall, 1991). Chemokine production is mediated at the transcriptional level and the amount of mRNA is influenced by the regulation of its stability. Many chemokines harbour AU rich sequences at the 3'-end, non-translated region, the presence of which has been shown to be a rate defining step in the degradation of mRNA (Brewer & Ross, 1988).

In terms of protein structure, the basic nature of chemokines allows binding to negatively charged proteoglycans including heparin (CC chemokines MIP-1α and β are an exception since they are acidic although still bind heparin (Wolpe *et al.* 1988)). This has lead to the idea that chemokines might be mobilised along the extracellular matrix and in this way could provide a solid phase chemotactic gradient toward a site of inflammation; so-called haptotaxis (Witt, 1994). In this respect, the standard assay to

characterise the chemokines *in vitro*, namely chemotaxis using some form of Boyden chamber, may be somewhat flawed since cells migrate along a fluid phase (Falk *et al.* 1980). However, it is possible that chemokines are mobilized to the polycarbonate filters through which the cells migrate.

The presence of the four cysteine motif is crucial for both CC and CXC chemokines allowing the formation of disulphide bridges between cysteines 1-3 and 2-4 which is essential for biological activity (Matsushima & Oppenheim, 1989). It is unclear how this picture might accommodate the 'C' chemokine, lymphotactin, which lacks cysteines 1 and 3 but still retains lymphoattractant activity (Kelner *et al.* 1994). Overall sequence homology between the CXC and CC families is low (20-40 %) (Oppenheim *et al.* 1991) and in this respect is it curious that they form similar monomeric tertiary structures; a single C-terminal α helix packed against a three-stranded antiparallel β sheet (Wells *et al.* 1996). It has been suggested that hydrophobic sequences on the monomer surface are conserved in both families but that the exact distribution of these residues conveys receptor selectivity for ligands. In contrast, dimeric structures are very different between the two families (Wells *et al.* 1996), and so the assessment of quaternary structure at physiological concentrations (<100 nM) is important. It has been suggested that the monomeric form is the active form, since the concentration of most chemokines *in vivo* is within the nanomolar range (Paolini *et al.* 1994). In support of this, the IL-8 analogue N-Methyl-Leu-25 is unable to dimerise and yet has a similar potency to wild type IL-8 *in vitro* (Wells *et al.* 1996).

1.6.8.2. CXC chemokines

The first chemokine to be identified and characterised was IL-8 by Yoshimura *et al* (1987a) as a major neutrophil chemoattractant component from IL-1-stimulated human monocytes. However, some years earlier Deuel *et al* (1977) reported the sequence of platelet factor 4 (PF4), which was later described as a neutrophil and monocytic chemoattractant activity (Deuel *et al.* 1981). Although structurally this chemokine is indeed a member of the CXC subfamily, the studies performed by Deuel used purified and not recombinant PF4. It is possible that these samples were contaminated with other chemokines since recombinant PF4 does not attract these cell types (J. Westwick, personal communication). Moreover, PF4 lacks the ELR motif seemingly required for neutrophil migration (see below).

Monocytes are rich sources of CXC chemokines; as well as IL-8, IP10 (Luster *et al.* 1985) and monokine induced by IFNγ (mig-γ, Farber 1993) were identified as products of IFNγ-stimulated macrophages. Other members of the family include MGSA/*gro* α (Richmond *et al.* 1988), *gro* β, *gro* γ, (Haskill *et al.* 1990; Iida & Grotendorst, 1990), NAP-2 and CTAP-III which arise from the N terminal processing of platelet basic protein (Walz *et al.* 1989), endothelial-cell-derived neutrophil-activating protein (ENA-78, (Walz *et al.* 1993) and granulocyte chemotactic protein-2 (GCP-2, Proost *et al.* 1993).

A feature of the CXC chemokines is that N terminal modifications are common place, for example IL-8 is predominantly secreted as a 77 amino acid protein from endothelial cells, denoted $[ala\text{-}IL\text{-}8]_{77}$, where as monocytes and T lymphocytes release the N-terminally reduced $[ser\text{-}IL\text{-}8]_{72}$ form. Both proteins are able to stimulate neutrophils but full activation of IL-8 requires cleavage by thrombin to the 72 amino acid form (Hebert

et al. 1990) which induces chemotaxis (Yoshimura *et al.* 1987b), calcium mobilisation and, in the presence of cytochalasin B, azurophil granule release at nanomolar concentrations (Peveri *et al.* 1988). Colditz *et al* (1989) demonstrated relevance *in vivo* as oedema and neutrophil accumulation were evident at 4 hours post-intradermal injection of rhIL-8 into rabbits. The neutrophil chemoattractant ability of IL-8 requires the presence of the N-terminal tripeptide sequence Glu-Leu-Arg ($E_4L_5R_6$) preceeding the first cysteine (Hebert *et al.* 1991).

There is some evidence to suggest that IL-8 plays a role in allergic inflammation. This chemokine can induce small but significant increases in $[Ca^{2+}]_i$ in normal human eosinophils (Kernen *et al.* 1991) and possesses weak chemoattractant activity (Erger & Casale, 1995). This response is increased using eosinophils from asthmatic subjects (Warring *et al.* 1993) which may be a result of IL-5 priming of the cells *in vivo* since the preincubation of normal eosinophils with IL-5 *in vitro* potentiates IL-8-induced stimulation (Schweizer *et al.* 1994). IL-8 also causes the migration of selective T lymphocytes *in vitro* at picomolar doses (Larsen *et al.* 1989, Bacon *et al* 1989). Many of these observation are supported by *in vivo* evidence. Leonard *et al* (1990) observed basophil, lymphocyte and neutrophil accumulation in dermal-injected sites, whilst Burrows *et al* (1990) reported lymphocyte and eosinophil accumulation in the BAL of guinea pigs given intraperitoneal injections of IL-8. However, Collins *et al* (1993) observed that accumulation of ^{111}In-labelled eosinophils into IL-8 injected skin sites in the guinea pig was delayed until 1-2 hours post injection. This compared to an immediate accumulation in ZAP-injected sites suggesting that an intermediary player released by IL-8, and not IL-8 alone, may be mediating eosinophil migration. IL-8 is released by numerous cell types that are associated with allergic inflammation, including alveolar macrophages (Carre *et al.* 1991), pulmonary epithelial cells (Standiford *et al.*

1990a) and notably, the eosinophil (Braun *et al.* 1993) suggesting the establishment of a positive feedback mechanism.

1.6.8.3. CC chemokines

The CC chemokines have been extensively studied in association with allergic asthma by virtue of their preferential recruitment of eosinophils, monocytes, basophils and a variety of T cell subsets. In particular, RANTES, eotaxin, MCP-1 and 3, MIP-1α have been widely implicated (Lukacs *et al.* 1996a) and a summary of their activities is provided below.

CC Chemokine	Human target cell	Reference
RANTES	Eosinophil	(Rot 1992)
	Monocyte	(Schall 1990)
	T lymphocyte ($CD4^+/CD8^+$)	(Schall 1990)
		(Turner 1995)
	Basophil	(Kuna 1993)
		(Bacon 1994)
Eotaxin	Eosinophil	(Jose *et al.* 1994b)
MCP-1	Monocyte	(Yoshimura 1989b)
	Basophil	(Alam 1992b)
	T cell	(Taub 1995)
MCP-3	Eosinophil	(Weber 1995)
	Monocyte	(Van Damme 1992)
	T cell	(Taub 1995)
	Basophils	
MIP-1α	Eosinophil	(Rot 1992)
	$CD8^+$ T and B lymphocyte	(Schall 1993),
	Monocyte	(Taub 1993)

Table 1.2. Summary of CC chemokines implicated in cell recruitment in allergic inflammation. All induce chemotaxis of their respective cell types, except for basophils which undergo histamine release.

Monocyte chemotactic proteins (MCP)

The CC chemokines were originally described as attractants of monocytes but not neutrophils (they lack the ELR motif of the neutrophil attracting CXC chemokines) and in this respect, the monocyte chemotactic peptides are prototypes (Oppenheim *et al.* 1991). MCP-1 was identified by Matsushima *et al* (1989) and Yoshimura *et al* (1989b) in supernatant from stimulated PBMCs and a monocytic cell line respectively and assumed to be the homologue of the previously reported murine protein JE from PDGF-stimulated 3T3 fibroblasts (Cochran *et al* 1983, Rollins *et al.* 1988). In 1992, Van Damme and colleagues (1992) purified two related chemokines with identities of 62 and 73% to MCP-1 from an osteosarcoma cell line, labelled MCP-2 and MCP-3 respectively. MCP-1 is a potent stimulator of chemotaxis (Yoshimura *et al.* 1989a), calcium influx and respiratory burst in human monocytes and also regulates adhesion molecule expression in these cells (Jiang *et al.* 1992). In comparison to other CC chemokines however, MCP-1 is not a chemoattractant for eosinophils (Rot *et al.* 1992).

MCP-1 is one of the most potent mediators of histamine release from basophils being active at nanomolar concentrations and comparable to anti IgE or C5a (Alam *et al.* 1992b). RANTES, and to a lesser extent MIP-1α and β, CTAP III and the related NAP-2 can also stimulate a similar response (Kuna *et al.* 1992, 1993). Alam and colleagues (1988) described the presence of a substance produced by mononuclear cells that inhibited histamine release from basophils, later identified as IL-8 by Kuna *et al* (1991) which does not have any significant histamine-releasing ability. Alam *et al* (1992a) also reported RANTES as having a similar inhibitory activity towards MCP-1-induced histamine (Alam *et al.* 1992a). One explanation for these inhibitory effects could be that pretreatment of basophils with a certain chemokine will desensitise the histamine-

releasing capacity of another (Kuna *et al.* 1992). It is also noteworthy that MCP-1 and RANTES induce the migration of basophils *in vitro* (Bischoff *et al.* 1993).

Macrophage inflammatory protein-1

Macrophage inflammatory protein-1α and β were originally isolated from the LPS stimulation of a mouse macrophage cell line (Wolpe *et al.* 1988; Davatelis *et al.* 1988) although the cDNA to the human homologue of MIP-1α (LD78) had actually been cloned a few years earlier from PMA-treated tonsillar lymphocytes by Obaru *et al* (1989). It would now appear that both forms of MIP are expressed in T cells, B cells, and activated neutrophils (Kasama *et al.* 1993). MIP-1α is able to stimulate chemotaxis and degranulation of human eosinophils over a nanomolar range and is particular potent at inducing changes in $[Ca^{2+}]_i$ compared to RANTES or IL-8 (Rot *et al.* 1992). MIP-1α also demonstrates chemotactic activity for B cells (Schall *et al.* 1993) as well as $CD4^+$ and $CD8^+$ T lymphocytes (Taub *et al.* 1993), and upregulates IL-1, TNFα and IL-6 production from macrophages (Fahey *et al.* 1992). It can induce increases in $[Ca^{2+}]_i$ in neutrophils, but this does not appear to translate into a functional role such as migration (McColl *et al.* 1993). Subcutaneous injection of MIP-1α into the footpads of mice induces an immediate swelling followed by a long-lasting monocytic infiltrate (Alam *et al.* 1994b). Despite the fact that hMIP-1β bears 70% amino acid homology to hMIP1α and is structurally very similar, MIP-1β does not stimulate human eosinophils (Rot *et al.* 1992). The discrepancy between homology and activity of these two proteins is further supported by their ability to attract different lymphocyte populations (Schall *et al.* 1993).

The MIP proteins are somewhat distinct from other CC chemokines since they have a number of other roles in addition to chemoattraction. MIP-1α induces pyrogenic activity in the rabbit which is prostaglandin independent and cannot be blocked by cyclooxygenase inhibitors (Davatelis *et al.* 1989). Furthermore, MIP-1α is reported to inhibit haematopoiesis by preventing colony formation of immature progenitors, although IL-8 and MCP-1 may also induce similar effects (Broxmeyer *et al.* 1990).

RANTES

Schall *et al* (1988) originally identified RANTES (an acronym for Regulated on Activation Normal T cell Expressed and Secreted) in 1988 as a product of IL-2 dependent, antigen-driven human T cell clones during a search to identify novel cDNA clones present in T cells, but not B cells. The cDNA encodes a 91 amino acid protein that includes a cleavable 23 amino acid hydrophobic signal sequence at the N-terminus and a mature secreted protein of 7.9 kDa (Schall *et al.* 1988). The RANTES protein is highly basic (pI of around 9.5) and contains no sites for N-glycosylation. A murine RANTES cDNA has also been cloned and encodes a mature protein with 85 % amino acid homology to its human counterpart (Schall *et al.* 1992).

RANTES was distinct from the early so called 'sis' or (small inducible, secreted) proteins in showing an inverse pattern of transcript regulation in T cells (ie. RANTES expression decreased in activated T cells whilst MIP-1α increased) (Schall *et al.* 1988; Oppenheim *et al.* 1991). Studies of the *Syca5* gene encoding mRANTES suggest that these differences in inducibility may be explained by the absence of a 10 nucleotide core sequence in the promotor region of the RANTES gene which is present in other CC chemokines (Danoff *et al.* 1994). It is interesting that RANTES also lacks AU rich

sequences in the 3' untranslated region of its mRNA (Schall *et al.* 1988), the presence of which has been shown to reduce the half life of other cytokines (Brewer & Ross, 1988).

The biological activity of RANTES

The characterisation of human RANTES suggested that it might play a pivotal role in the pathogenesis of allergic inflammation. The protein attracts eosinophils over a nanomolar range (Kameyoshi *et al.* 1992, Rot *et al.* 1992; Alam *et al.* 1993), as well as CD45RO T cells (primed T helper cell involved in memory function) and monocytes (Schall *et al.* 1990); a profile which mirrors the infiltrate found in the asthmatic lung (Azzawi *et al.* 1990). Rot *et al* (1992) ascribed a number of other eosinophil functions to RANTES including the ability to increase $[Ca^{2+}]_i$, and stimulate the release of ECP from cytochalasin B-treated cells although there was no evidence of LTC_4 formation. Furthermore RANTES induces respiratory burst in these cells (Rot *et al.* 1992, Kapp *et al.* 1994, Bourne *et al.* 1992). However, MIP-1α is more potent on a molar basis at inducing changes in $[Ca^{2+}]_i$ and ECP but less potent as a chemoattractant (Rot *et al.* 1992) whilst reports conflict on its ability to cause respiratory burst (Rot *et al.* 1992; Kapp *et al.* 1994, Bourne *et al.* 1992). Alam and colleagues (1993) demonstrated the ability of RANTES to induce a state of hypodensity, a feature of eosinophils from asthmatic subjects (Frick *et al.* 1989) and to upregulate the expression of CD11b at the same concentrations required to induce eosinophil chemotaxis. Later studies by Eibsawa (1994) could not reproduce this effect, although the authors did observe eosinophil transendothelial migration induced by RANTES which could be inhibited by antibodies against the β2 integrin, CD18. RANTES is also able to induce histamine

release from basophils (Kuna *et al.* 1992) and basophils from atopic patients demonstrate an increased responsiveness compared to nonatopics (Kuna *et al.* 1993).

The original characterisation of RANTES by Schall (1990) highlighted a role for RANTES as a chemoattractant of human monocytes and lymphocytes. These findings have since been supported by observation from a number of groups. RANTES-induced increases in $[Ca^{2+}]_i$ were reported by Vaddi *et al* (1994) in both human blood monocytes and the monocytic cell line THP-1, although MCP-1 was a more potent agonist. However, whilst monocytes readily underwent chemotaxis, THP-1 cells were prevented through an inability to achieve actin polymerisation and cell polarisation.

RANTES appears to mediate a number of lymphocyte cell functions. The $CD4^+$ T cell attractant activity of the protein has been described by a number of groups (Schall *et al.* 1990) although Taub and co-workers have suggested that $CD8^+$ subsets also migrate *in vitro* (Taub *et al.* 1993) and observed equal numbers of $CD4^+$ and 8^+ T cells in RANTES injected murine skin sites (Murphy *et al.* 1994). Studies by Turner *et al* (1996) indicate that RANTES is more than a lymphoattractant; this chemokine is able to induce T cell proliferation. Furthermore, important work by Dragic (1996) demonstrated that RANTES can inhibit HIV entry into $CD4^+$ T cells by blocking the CCR5 receptor. RANTES does not seem to be associated exclusively with either Th1 or Th2 phenotypes. Schrum and colleagues (1996) suggested that RANTES release from T cells was predominantly from Th1 clones secreting IL-2 and IFNγ. However, Kimata and colleagues (1996) have recently reported the ability of RANTES to induce IgE and IgG_4 production from B cells, an event associated with Th2 mediated responses (Del Prete *et al.* 1988). These immunoglobulins are selectively elevated in atopic subjects (Merret *et al.* 1984).

Cellular and tissue sources of RANTES

RANTES has been identified in a number myeloid cells such as T lymphocytes (Schrum *et al.* 1996) and monocytic cells (Devergne *et al.* 1994). However, studies by Kameyoshi *et al* (1992) demonstrated that thrombin-stimulated human platelets are a rich source of biologically active RANTES. Purification of the supernatant from these platelets and assessment of the eosinophil-chemotactic activity of resulting fractions identified two peaks which on sequencing were both found to be RANTES; one product with a slightly higher molecular weight due to glycosylation. This work allowed analysis of the native form of human RANTES. In addition, a number of authors have reported RANTES in a number of parenchymal/stromal cells, the expression of which *in vitro* frequently requires TNFα stimulation. These include dermal (Noso *et al.* 1995) and synovial (Rathanaswami *et al.* 1993) fibroblasts, smooth muscle cells (Jordan *et al.* 1995), epithelial (Kwon *et al.* 1995) and endothelial cells (Marfaing-Koka *et al.* 1995), epidermal and oral keratinocytes (Li *et al.* 1996), and human (Robson *et al.* 1995) and murine messangial cells (Wolf *et al.* 1993). Given the array of cells that produce RANTES, it is not surprising that mRNA for the protein is found in a number of whole tissues/biological fluids. These include the lung and BAL (Alam *et al.* 1996), kidney (Heeger *et al.* 1992) and the peritoneal fluid from subjects with endometriosis where the RANTES concentration correlated with the severity of disease (Khorram *et al.* 1993).

1.6.8.4. Chemokine receptors

It would appear that there is some functional overlap between the CXC and CC chemokines, for example eosinophil migration can be induced by IL-8 in some circumstances as well as by RANTES and MIP-1α. However redundancy between the families is minimised by distinct receptors for which the ligands do not cross-compete. In contrast, many investigators have observed intra-family sharing of receptors. Experimentally these patterns have been identified using radioligand binding displacement studies and calcium desensitisation profiles. The combined use of these techniques has proved useful since there are many examples where chemokine binding affinity does not predict functional activity of the cell.

In the case of the CXC chemokines, two receptors have been identified denoted IL-8-RA and IL-8-RB (Holmes *et al.* 1991; Murphy & Tiffany, 1991) and share an amino acid sequence homology of 77 % (Cerretti *et al.* 1993). IL-8-RA specifically binds IL-8 with high affinity, whilst IL-8-RB binds IL-8, MGSA/gro α and NAP-2 with equal affinity (Cerretti *et al.* 1993). The aforementioned ELR motif is thought to be essential for binding to these receptors and Clarke-Lewis *et al* (1993) have demonstrated that N terminal modification of PF4 to substitute ELR for the natural DLQ, allows binding to the IL-8 receptors and activation of neutrophils.

At the time of writing of this thesis, five human CC chemokine receptors each with murine homologues had been cloned This compares to one CC receptor at the commencement of the project, highlighting the vast expansion of this field. These are summarised below.

Receptor	Ligand	murine homologue	cellular distribution	Reference
CCR1	RANTES MIP-1α	mMIP1α	eosinophils, monocytes	(Proudfoot *et al.* 1995)
CCR2B	MCP-1 and 3	mJE-R	Monocytes	(Charo *et al.* 1994)
CCR3	Eotaxin, RANTES, MCP-3, MCP-4	mMIP1αRL2	Eosinophils, Monocytes	(Combadiere *et al.* 1995) (Daugherty *et al.* 1996)
CCR4	MIP-1α, RANTES, MCP-1	mCC CK_{4A}	B cell, monocyte, T lymphocyte	(Hoogewerf *et al.* 1996)
CCR5	MIP-1α, MIP-1β,RANTES	mMIP1α	monocytes, T lymphocytes	(Combadiere *et al.* 1996)

Table 1.3. Summary of the characterised CC chemokine receptors. Adapted from Power and Wells *et al* (1996).

Cloning of the first CC receptor (CCR1) from HL60 libraries was reported by Neote *et al* (1993) and Gao *et al* (1993) in close succession. When expressed in either human embryonic 293 cells or Xenopus oocytes, binding of RANTES and MIP-1α was observed but not to any CXC chemokines. MIP-1α was able to displace and abolish $[Ca^{2+}]_i$ responses induced by RANTES but RANTES was only able to reduce MIP-1α responses, suggesting partial desensitisation of the receptor by RANTES. Surprisingly this receptor binds MIP-1α with high affinity but RANTES with a much lower affinity, and yet these ligands induce $[Ca^{2+}]_i$ in transfected cells over a similar range (1-100 nM). Furthermore MCP-1 and MIP1-β only induce $[Ca^{2+}]_i$ changes at much higher doses, but bind the receptor with greater affinity than RANTES. Clearly the binding affinities of this receptor do not parallel the ability of the ligand to signal (Neote *et al.* 1993).

Neote (1993) and Gao (1993) reported a similar distribution for CCR1 on the myeloid presursor cells HL60, U937 and monocytic cell line THP-1, human B lymphocytes as well as dibutyryl cAMP-treated neutrophils and neutrophils from peripheral blood although its presence is unclear given the inability of either ligand to induce neutrophil migration. Furthermore the desensitisation of $[Ca^{2+}]_i$ responses in human eosinophils using MIP-1α and RANTES by Rot's group provided the first evidence of a shared receptor on these cells (Rot *et al.* 1992).

CCR1 demonstrates 32% identity with receptors for IL-8 and the orphan clone HUMSTSR (also known as fusin, LESTR and CXCR4), in comparison to 23% with the C5a receptor. In addition, an open reading frame, US28, of the human cytomegalovirus (CMV) also shares 33% identity with the MIP-1α/RANTES receptor which increases to 56 % over the acidic N terminal region. CMV is known to infect myeloid and lymphoid cells that might also express the chemokine receptor. It has been suggested that CMV infection could alter the immune response (thereby providing a more favourable environment for the virus) by competing for chemokine binding (Neote *et al.* 1993; Gao *et al.* 1993).

The notable exception to receptor exclusivity between the chemokine families is a receptor on the erythrocyte apparently identical to Duffy blood-group antigen which binds the malarial parasite *Plasmodium vivax*. This so-called Duffy antigen receptor (DARC) binds most chemokines with K_d values of 20-50 nM. Clearly there is no requirement for extravasation of erythrocytes, leading investigators to speculate that this receptor clears excess amounts of chemokines thus limiting leukocyte stimulation. However, this receptor is not restricted to erythrocytes, expression has also been detected on endothelial cells and T cells (Horuk *et al.* 1993).

All chemokine receptors identified are seven transmembrane spanning; a formation that allows the extracellular exposure of four conserved cysteine residues in the receptor sequence which are postulated to form disulphide bonds, an extracellular N terminus and intracellular C terminus. A highly conserved motif, DRYLAIVHA, within the third transmembrane domain 3 is present in all chemokine receptors, except DARC but not in other chemoattractant receptor such as C5a or fMLP (Neote *et al.* 1993). The two-step binding principle adopted by C5a to its 7TM receptor may be an applicable model of ligand/receptor interactions for the chemokines. The core of the ligand (region beyond first disulphide bond) binds the outer surface of the receptor, the N-terminus which may dictate receptor specificity. This interaction is probably stabilised through charge-charge interactions given the highly acidic nature of N-terminus of the chemokine receptors (which is uncommon in other G protein coupled receptors (Gao *et al.* 1993)) and the basic regions of the chemokine protein. Once orientated, the flexible amino terminus of the chemokine can position itself inside the receptor (as reviewed in Wells *et al.* 1996).

All chemokine receptors (except DARC) are coupled to GTP-binding proteins and so cellular responses induced by binding of their ligands are pertussis toxin-sensitive (Sozzani *et al.* 1991). Most signalling through such receptors occurs via dissociation of the α subunit from the $\beta\gamma$ subunits of the G-protein thus activating phospholipase $C_{\beta 2}$. The subsequent generation of inositol 1,4 ,5-triphosphate IP_3 and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2) allows a rapid and transient calcium release from intracellular stores as well as a cascade of phosphorylation and activation steps involving PKC and phospholipase D (Sozzani *et al.* 1994). The signal ultimately conveys to the cell motility, mediator production and/or oxidant production. Studies by Turner *et al* (1996) indicate that such a pathway does indeed exist and demonstrate

MCP-1-induced IP_3 generation in the monocytic cell line THP-1. However, earlier studies by Sozzani and colleagues (1993) failed to identify IP_3 or evidence of PIP_2 turnover in similar studies using human blood monocytes. Instead, they observed Ca^{2+} transients which could be blocked by Ni^{2+}, suggesting that increases in $[Ca^{2+}]_i$ were achieved through influx across the cell membrane. It is possible that chemokines also utilise other pathways. Turner *et al* (1995) demonstrated that RANTES-stimulation of human peripheral blood T lymphocytes induces increases in PI_3 kinase but observed no changes in $[Ca^{2+}]_i$.

Following the binding of ligand, receptors are internalised and are later recycled back to the surface of the cell (Kelvin *et al.* 1993). The response following cell stimulation can also be controlled by receptor desensitisation, which can occur on rapid successive exposure of the same ligand or another acting at this receptor. The basis of this phenomenon is phosphorylation of serine/threonine residues in the C terminus, a feature common to all G-protein-linked receptors (Hausdorff *et al.* 1992).

1.7. Introductory review of the literature

The aforementioned introduction concentrated on studies performed prior to the beginning of the my project. Publications arising during my studies have only been touched upon in this section. However this work is discussed fully in the context of my findings, in chapter 6.

1.8. Basis and aims of project

The use of the guinea pig as a suitable model of allergic inflammation centres around an ability to exhibit some of the features of the human asthmatic response and the development of eosinophilia in the lung. Clearly it is of great interest to assess the actions of chemokines and in particular, RANTES in this species. Murine RANTES (muRANTES) has been cloned, demonstrates 85 % amino acid identity to hRANTES and activates human blood monocytes (Schall *et al.* 1992). However there are examples of variations in chemokine activity between species, for example hMCP-1 is twice as potent at chemoattracting human blood monocytes than murine peritoneal macrophages (Luini *et al.* 1994). Furthermore, studies by Meurer *et al* (1993) indicate that although RANTES was able to induce eosinophil accumulation when injected into canine skin sites, no dermal responses were observed in lower species. To gain an accurate assessment of the role of RANTES in the guinea pig necessitates the use of the same species protein.

The aims of this project were therefore as follows:

1. To establish the presence of RANTES messenger RNA in the guinea pig lung
2. To determine a pattern of spatial and temporal expression of RANTES in the guinea pig following ovalbumin challenge
3. To purify recombinant guinea pig RANTES from transfected *E. coli*
4. Characterisation of the biological activity of guinea pig RANTES (gpRANTES) and hRANTES *in vitro* towards eosinophils and macrophages.
5. Characterisation *in vivo* to assess the migratory response towards gpRANTES in the guinea pig lung and skin

CHAPTER 2. MATERIALS AND METHODS

Unless indicated, all reagents were purchased from Sigma Chemical Co., Poole, UK.

REAGENT	SUPPLIER
Acrylamide mini gels (precast; 4-20%)	Novex, San Diego, USA
L-Agar	Difco, West Molesey, Surrey, UK
Agarose MP	Boehringer Mannheim, Mannheim, Germany
Anti-DIG-AP-conjugated Fab fragments	Boehringer Mannheim, Mannheim, Germany
Ampli Taq (with x10 buffer)	Perkin Elmer, Roche Molecular Systems, NJ, US
Bacto tryptone	Difco, West Molesey, Surrey, UK
Bacto yeast extract	Difco, West Molesey, Surrey, UK
Bio-Rad Mini protean system	Bio-Rad Laboratories, Richmond, USA
Bio-Rad protein assay	Bio-Rad Laboratories, Richmond, USA
Blocking stock for nuclei acid hybridisation	Boehringer Mannheim, Mannheim, Germany
Boric acid	BDH laboratory supplies Ltd, Lutterworth, UK
Bronchial epithelial growth medium	Clonetics Corporation, Walkersville, MD, USA
Calcium chloride	Fisons, Loughborough, UK
Centrifuge tubes 15&50ml (polypropylene)	Falcon for Becton Dickinson, Franklin Lakes,USA
Chromatographic media	Pharmacia Biotech, Uppsala, Sweden
Collagen- (rat tail) coated 6 well plates	Costar, California, USA
Collagenase/Dispase	Boehringer Mannheim, Mannhein, Germany
Coomassie Blue Reagent-250	Bio-Rad, Hemel Hempstead, UK
Diff-Quik	Dade AG, Dudingen, Switzerland
Digitonin	BDH laboratory supplies Ltd, Lutterworth, UK
DIG-11-dUTP	Boehringer Mannheim, Mannheim, Germany
DNA molecular weight ladder (100bp)	Gibco Life Technologies Ltd, Paisley, UK
DNA - DIG labelled DNA of known amount	Boehringer Mannheim, Mannheim, Germany
ECL (Enhanced Chemiluminescence) reagents	Amersham Int. PLC, Little Chalfont, UK
E. coli	Stratagene, Cambridge, UK
ELISA 96 well plates (Nunc 'Maxisorp')	Gibco Life Technologies Ltd, Paisley, UK
Endoproteinase Arg C	Boehringer Mannheim, Mannheim, Germany
Eotaxin cDNA labelled oligoprobe	R&D systems, Abingdon, Oxon, UK
Ethanol	Fisons, Loughborough, UK
Ethidium bromide	Sigma, Poole, UK

Euthatal (pentobarbitone)	Rhône Merieux, Dublin, Eire
FCS	Gibco Life Technologies Ltd, Paisley, UK
Formaldehyde	Fisons, Loughborough, UK
Formamide	BDH laboratory supplies Ltd, Lutterworth, UK
Fura-2AM	Calbiochem, La Jolla, US
Gentamycin	Gibco Life Technologies Ltd, Paisley, UK
Glacial acetic acid	BDH laboratory supplies Ltd, Lutterworth, UK
Glycerol	BDH laboratory supplies Ltd, Lutterworth, UK
Guanidinium thiocyanate	Fluka, Buchs, Switzerland
HBSS x10	Gibco Life Technologies Ltd, Paisley, UK
Heparin ('Monoparin')	CP Pharmaceuticals Ltd, Wrexham, UK
Histological stains	BDH laboratory supplies Ltd, Lutterworth, UK
Horse radish peroxidase-conjugated goat anti-mouse and goat anti-rabbit Abs	Bio-Rad Labs, Hemel Hempstead, UK
Horse serum	Gibco Life Technologies Ltd, Paisley, UK
IFNγ	GlaxoWellcome, Stevenage, UK
IMS	BDH laboratory supplies Ltd, Lutterworth, UK
^{111}Indium chloride and ^{125}Iodine-human serum albumin	Amersham Int., Bucks, UK
Interleukin-5	GlaxoWellcome, Geneva, Switzerland
Interleukin-8	Sandoz, Vienna, Austria
JH4-Clone 1 fibroblast, guinea pig cell line	American Type Culture Collection, Rockville, USA
Ketamine	Parke Davis Veterinary, Pontypool, UK
Lambda DNA markers	Northumbria Biologicals Ltd, Cramlington, UK
LB medium	Difco, West Molesey, Surrey, UK
Lymphoprep	Nycomed, Oslo, Norway
Marvel non-fat milk	Premier Brands UK Ltd, Stafford, UK
Methyl green histological stain	Vector Laboratory, Peterborough, UK
MIP-1α/β	Peprotech, London, UK
M-MLV RTase	Promega, Madison, US
Nitrocellulose membrane for protein transfer	Bio-Rad Laboratories, Richmond, USA
Nucleotides (Ultrapure dNTP set)	Pharmacia Biotech, Uppsala, Sweden
PAF	Bachem, Bubendorf, Switzerland
Paraformaldehyde	BDH laboratory supplies Ltd, Lutterworth, UK
PBS x10 (Dulbecco's)	Gibco Life Technologies Ltd, Paisley, UK
Penicillin	Gibco Life Technologies Ltd, Paisley, UK
Percoll	Pharmacia Biotech, Uppsala, Sweden
Phenol/chloroform	Camlab, Cambridge, UK
Primers for DNA amplification	Perkin Elmer Cetus, manufactured by Roche Molecular Systems, Inc., NJ, US
Propan-2-ol (isopropanol)	Fisons, Loughborough, UK
Protogel	National Diagnostics, Atlanta, US
QIAquick gel extraction kit	Quigen GmbH, Hilden, Germany

Random hexamers (pd(N)6)	Boehringer Mannheim, Mannheim, Germany
Restriction enzymes	New England Biolabs, Beverly, US
Riboprobe Gemini System	Promega by Roche Mol. Systems Inc, Branchburg, US
RNAguard-RNase inhibitor	Promega by Roche Mol. Systems Inc, Branchburg, US
RNAsol B	ams Biotechnology, California, USA
RNasin-RNase inhibitor	Promega by Roche Mol. Systems Inc, Branchburg, US
RPMI medium	Gibco Life Technologies Ltd, Paisley, UK
Sodium Bicarbonate (7.5%)	Gibco Life Technologies Ltd, Paisley, UK
Sodium chloride	Fisons, Loughborough, UK
Streptomycin	Gibco Life Technologies Ltd, Paisley, UK
Tween-20	BDH laboratory supplies Ltd, Lutterworth, UK
Urea	Fluka, Buchs, Switzerland
WEB 2086	Boehringer Mannheim, Mannheim, Germany
Xylazine	Bayer, Leverkusen, Germany

2.1. DETECTION OF CHEMOKINE mRNA IN THE GUINEA PIG LUNG

2.1.1. Preparation of digoxigenin-labelled probes

2.1.1.1. Preparation and purification of chemokine cDNA

Transformation of E. coli XL-1 Blue cells by electroporation. cDNAs encoding both orientations of full length guinea pig chemokines were subcloned by T. Yoshimura (Laboratory of Immunobiology, NCI-FCRDC, Frederick, Maryland, USA) into the multicloning region of pBluescriptII SK- plasmid. All subsequent procedures were carried out at Bath University by myself.

Sufficient *E. coli* to give an OD_{600} of 0.5-1.0 in 500 ml were pelleted by centrifugation at 4000 g for 15 min at 4 °C. The ionic strength of the cells was reduced by sequential washing in 500 ml sterile water, 250 ml sterile water and 10 ml of 10% glycerol, all at 4°C. Final resuspension was in 0.5 ml of 10% glycerol, and aliquots (40 µl) were snap frozen in a dry-ice/methanol bath and stored at -70 °C. Plasmid DNA (0.5 µg) was mixed with a thawed aliquot of electrocompetent *E. coli* on ice, transferred to a sterile pre-chilled 0.2 cm electroporation cuvette and placed in a cuvette carrier of a Gene Pulser and Pulse Controller (Bio-Rad Laboratories, Richmond, CA, US). The cells were subjected to a single 2.5 kV pulse, with 25 µ Farads capacitance and 200 Ohm resistance, to produce a nominal time constant for the capacitor discharge of 5 ms. Following electroporation, cells were allowed to recover by constant shaking in 1 ml recovery medium at 37 °C for 1 h. Transformed cells were selected by spreading onto L-agar plates containing ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml) to achieve single-colony density, and grown at 37 °C overnight. A single colony was transferred using sterile technique into a 10 ml LB broth containing ampicillin (100 µg/ml) and tetracyclin (12.5 µg/ml) and cultured at 37 °C overnight.

Small scale preparation of bacterial plasmids (Miniprep). Cells from the 1 ml of culture were pelleted by centrifugation (13,000 g, for 1 min at 4 °C), the supernatant removed and DNA extracted by standard miniprep extraction according to Sambrook and Manniatis (1989). Briefly, the cells were resuspended in 100 µl of solution I (see Appendix I) by vigorous vortexing and lysed with 200 µl of freshly prepared solution II, mixing by gentle inversion. The contents were placed on ice for 10 min then 150 µl of ice-cold solution III was added and mixed by vortexing. The mixture was centrifugated (13,000g, 5 min) and the supernatant transferred to a clean 1.5 ml tube. Contaminating proteins were removed by two cycles of phenol-chloroform extraction (0.1 M Tris-saturated phenol/chloroform pH 8) and centrifugated at 13,000 g for 2 min. Plasmid DNA was precipitated from the aqueous layer by addition of 0.1 volume of 3 M sodium acetate, pH 5.5, and 2.5 volumes of absolute ethanol and then freezing in a dry ice/methanol bath. The DNA was pelleted by centrifugation (13,000g, 20 min at 4 °C) and washed with 70 % ethanol. Air dried pellets were resuspended in 50 µl of double-distilled water.

Analysis by restriction enzyme digestion. Enzyme restriction of the pBluescriptII SK- plasmids was used to confirm the presence of chemokine cDNA inserts and thus that electroporation and colony selection procedures had been successful. Chemokine cDNA inserts were removed by digesting 1 µg of plasmid DNA with suitable restriction enzymes selected to cut either side, but not at points within the sequence. Each plasmid was digested with 0.5 µl of enzyme in a 10 µl reaction in the presence of enzyme-specific buffer and RNase A.

Analysis by electrophoresis. Digestion products were separated by electrophoresis using Bio-Rad DNA sub-cells. Sufficient agarose for a 1 % gel in 30 ml was dissolved in 0.5x TBE and ethidium bromide added to a final concentration of 0.5 μg/ml before casting. Once set, gels were submerged in 0.5x TBE and DNA samples (0.5 μg/lane) were loaded with 0.2 volume 5x DNA loading buffer. Electrophoresis was carried out at 100-150 V for 30 min, and the DNA bands were visualised by UV light using a Hoefer Mighty Bright UVTM 25 transilluminator (Hoefer, San Fransisco, USA). Photographs were taken using Polaroid 55 film in a Polaroid CU-545 land film holder (Polaroid Corporation, Cambridge, MA, USA).

2.1.1.2. Preparation of full length PCR-generated cDNA probes

Plasmid DNA was amplified using a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer, CA, USA). cDNA (5 μg) was added to a reaction mixture containing; x1 PCR buffer, 200 μM each of dATP, dCTP, dGTP, 180 μM dTTP and 20 μM DIG-11-dUTP, 1 μM of both forward and reverse primers (see sequences in section 2.2), 0.025 U/μl *Ampli*Taq DNA polymerase, and double-distilled water to achieve a final reaction volume of 100 μl. The reaction was initially heated to 95 °C for 1 min, pausing at 30 sec to allow addition of 2 mM (final) of Mg^{2+}. Cycle conditions were set as denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec and extension at 72 °C for 15 sec for 30 cycles followed by a prolonged extension at 72 °C for 6 min. DNA was precipitated as described above and resuspended in 100 μl of water. The amount of DIG-labelled probe generated was estimated by dot blot analysis and comparison to a sample of DIG-labelled DNA of known concentration (Boehringer Mannheim). The specificity of amplification of pBluescript SK- plasmids containing the required

chemokine cDNA insert was confirmed by the presence of fragments of expected size using electrophoresis. Labelled probe was stored in aliquots at -20°C.

2.1.1.3. Preparation of single-stranded DIG-labelled riboprobes

pBluescriptII SK- plasmids (10 μg) containing either full length gpRANTES or eotaxin cDNA subcloned into the EcoRII site and EcoRI/XbaI site respectively, were linearised overnight with restriction enzymes in 100 μl reactions containing the appropriate buffer and 5 μg of RNase A. The enzymes were selected to allow linearisation on either the T7 or T3 polymerase-side of the insert to enable generation of antisense (complementary to mRNA) or sense (identical sequence to mRNA) probes respectively.

To minimise contamination of non-linearised plasmid, digestion products were separated by electrophoresis using a 1.5 % agarose gel (1 μg DNA per lane) containing 0.5 mg/ml ethidium bromide. Gel bands containing linearised DNA were removed using a sterile scalpel and DNA isolated using QIAquick Gel Extraction kit purification columns according to the manufacturer's instructions. DNA was precipitated as described above and resuspended at approximately 1 μg/10 μl in double-distilled DEPC-treated water.

Purified linearised cDNA (1 μg) was transcribed using Riboprobe Gemini System. Each reaction contained the following: 1x transcription buffer, 10 mM DTT, 0.5 mM each of dATP, dCTP, dGTP, 0.325 mM dUTP, 0.175 mM DIG-11-UTP, 1 μg plasmid, 50 U RNasin ribonuclease inhibitor, 10 U T7 or T3 polymerase and double-distilled water to a final volume of 50 μl. The reaction was incubated for 90 min before removal of the template by addition of RQ1 DNase (1 U/mg of DNA) for 15 min at 37 °C. This

reaction was quenched by 2 µl 0.5 M EDTA pH 5.5, and the cRNA was precipitated with 0.1 volume of 3 M sodium acetate and 3 volumes of ethanol at -70 °C for 1 h. cRNA was recovered by centrifugation at 12,500 g for 20 min, the pellet washed in 70 % ethanol and resuspended in 50 µl sterile DEPC-treated water.

The efficiency of DIG-labelling was assessed by dot blot comparison with dilutions of a labelled DNA sample of known concentration (Boehringer Mannheim).

2.1.2. Treatment of animals and preparation of lung tissue for analysis

Dunkin Hartley guinea pigs (300-400 g, male or female) were sensitised with 10 µg i.p. ovalbumin (OA) and aluminium hydroxide as an adjuvant and boosted again on day 14. On day 24, the animals were pretreated with mepyramine (1 mg/kg i.p.) before challenge with aerosolised ovalbumin (0.1 %) for 30 min. Similar sensitisation and challenge regimes have previously been demonstrated to induce IgE production in the guinea pig (Andersson 1980). Animals were overdosed with Euthatal (pentobarbitone) at various times post-treatment and lung tissue removed from each animal immediately (preparation of animals by A.-M. White). Approximately 0.5 cm^3 pieces were preserved by snap freezing in liquid nitrogen and storing at -70 °C. For *in situ* hybridisation and histological analysis, 1 cm^3 sized lung segments were removed from some animals, fixed in formol buffered saline for 18 h and later embedded in paraffin wax using a Tissue-Tek VIP Processor (Tissue Tek, Hants, UK). Sections of the embedded tissue were cut at 5 µm using a rotary microtome (Leica Instruments GmbH, Hussloch, Germany) and collected on APES-precoated microscope slides. Slides were APES-coated by cleaning in concentrated nitric acid for 30 min, rinsing in distilled water for 2

h and acetone for 10 min before baking at 180 °C for a further 2 h. They were then dipped in 2 % APES in acetone, rinsed with DEPC-treated water and air dried.

2.1.3. Isolation and growth of primary cells from guinea pig lung

The establishment of primary cells allows chemokine expression in individual cell types to be established and time course patterns following stimulation *ex vivo* to be determined. Fibroblasts have been grown as adherent cells that can be readily passaged from a number of tissues including human lung (Rolfe *et al.* 1992). Primary fibroblast-like cells were grown from guinea pig lung digests. One lobe of lung was removed from an animal killed by an overdose with Euthatal. The tissue was bathed in DMEM with Ham's F12 supplement using sterile technique and cut into small pieces before incubation in collagenase at 1 mg/ml (type II from Clostridium histolyticum) in the same medium for 1 h at 37 °C. The digest was pipetted vigorously, and large undigested fragments were allowed to settle. The suspension was spun at 300 g for 10 min and the pellet resuspended in DMEM/Hams F12 containing 10 % FCS, penicillin (100 μg/ml) and streptomycin (100 U/ml) before plating into T25 flasks. The cells were grown to confluence and passaged with trypsin (0.02 mg/ml) and EDTA (0.05 mg/ml) up to passage 6. For purposes of comparison, separate cultures of a guinea pig lung fibroblast cell line, JH4-Clone 1 (purchased from the American Type Culture Collection) were grown using the same medium.

Confluent cultures were stimulated with conditioned media from LPS-treated guinea pig peritoneal macrophages. This was prepared by the stimulation of $2x10^6$/ml MØ with 1 μg/ml LPS for 2 h, followed by the resuspension of the cells in fresh DMEM/Ham's F12 medium which was harvested at 24 h for use as a stimulant. In some experiments, the

supernatant from Con A-stimulated guinea pig spleen cells ($1x10^7$/ml cells treated with 1 μg/ml Con A for 24 h) was used at a 1/10 dilution in conjunction with the macrophage supernatant.

Much effort was made to isolate primary lung epithelial cells since these cells appear to be important in chemokine production, at least in humans (Kwon *et al.* 1995). Tracheas were removed from guinea pigs killed by Euthatal overdose and washed in RPMI 1640 medium prewarmed to 37 °C. Each trachea was immersed in 5 ml of RPMI supplemented with penicillin (100 μg/ml) and streptomycin (100 U/ml) and containing 1mg/ml collagenase (type II as before) and incubated for 30 min. The lumen of each trachea was repeatedly flushed through with the same medium using a 1 ml syringe and the cell suspension centrifuged at 350 g for 10 min. The pellet was resuspended in Bronchial Epithelial Cell Growth Medium (BEGM, see Appendix 2 for details) purchased from Clonetics Corporation (Walkersville, MD, USA). Cells were grown on rat tail collagen-coated wells of 6 well plates (Co-star) using one well per trachea.

2.1.4. In Situ Hybridisation methodology

2.1.4.1. Pretreatment of tissue sections

Guinea pig lung sections were analysed by *in situ* hybridisation based on a protocol by Vignaud *et al.* (1994). Initially paraffin-embedded sections were dewaxed by immersion into xylene (2x5 min) then hydrated through graded alcohols: 100% IMS (2x2 min), 90% (1 min), 85% (1 min) to 70% (1 min). Sections were permeabilised by immersion in 0.3% Triton-X in PBS for 10 min followed by a brief wash in PBS. Further permeabilisation was achieved by exposure to 2 mg/ml (15 units/mg) proteinase K solution extracted from Tritirachium album (Sigma, Poole, UK) in 20 mM Tris-HCl

and 1 mM EDTA, pH 7.2, for 10 min at 37 °C, which was quenched by immersion in 2 mg/ml glycine in PBS. The sections were refixed in 4 % paraformaldehyde solution for 10 min, before incubation in a stirring bath containing 0.1M triethanolamine solution (pH 8) with 6% v/v acetic anhydride added immediately prior to use. This step acetylates the sections and reduces non-specific binding due to charge interactions. Endogenous alkaline phosphatase activity was minimised by immersion for 15 s in 20% acetic acid, precooled to 4 °C. Section were rinsed in PBS before dehydration back though the graded alcohols to 100% IMS (2x1 min) and air-dried.

2.1.4.2. Hybridisation conditions

Section were prehybridised by addition of 100 µl per slide of prewarmed hybridisation buffer containing 50% formamide, 5x SSC (from a x20 stock containing 3 M NaCl and 0.3 m trisodium citrate at pH7.0), 10% dextran sulphate, 100 µg/ml heat denatured salmon sperm DNA, and 1x Denhardt's solution, for 30 min. This buffer was then removed before replenishing the sections with 50 µl of 1 ng/ml probe diluted in fresh hybridisation buffer and covering with a small piece of Parafilm approximately the same size as the section, to minimise evaporation. The slides were then incubated overnight at 50 °C in a sealed container lined with filter paper soaked in 2x SSC. Suitable stringency washing conditions for the removal of non-specific binding of probe to section were found to be 5x SSC (2x5 min) followed by 2x SSC (2x5 min).

2.1.4.3. Immunological detection of DIG-labelled probes

Section were washed in TBS containing 0.05% Tween-20 for 5 min and blocked with 1% albumin (bovine fraction V) in the TBS/Tween buffer for 30 min. Slides were rinsed with 2% normal sheep serum in TBS/Tween briefly, before incubation with 7.5 U/ml anti-DIG-AP Fab fragments for 2 h. Excess antibody was removed by sequential washing in TBS/Tween (2x10 min) and TBS (2x10 min), and the remaining antibody detected by addition of NBT/BCIP alkaline phosphatase substrate (100 µl per slide; see Appendix 1). After overnight incubation, the substrate was removed by washing with double-distilled water and the sections counterstained with methyl green for 10 min. To avoid removal of the counterstain, the section was blotted with damp blotting paper, air-dried before briefly rinsing in xylene and mounted in non-aqueous mountant using a Tissue Tek Automatic Coverslipper (Tissue Tek, Hants, UK).

For each lung, a separate slide was prepared and stained with haematoxylin and eosin to aid histological analysis. These sections were dewaxed in xylene, hydrated through the graded alcohols to water before immersion in alum haematoxylin (7.5 g/L) for 2-3 min. After a brief rinse in tap water the sections were rinsed in acid-alcohol (1% HCl in 70% ethanol) to allow differentiation of the colour and stained with 1% aqueous eosin Y. The slides were then washed in tap water, dehydrated through the alcohols, cleared in xylene and mounted. All tissue and slide preparation and section staining was performed by myself.

2.1.5. *Northern blot analysis methodology*

2.1.5.1. Extraction of total RNA

A number of methods based on work by Chomczynski and Sacchi (1987) were employed to isolate total RNA from whole guinea pig lung since successful methodology was not well documented in the literature. In initial experiments, frozen tissue (50 mg) was homogenised in the presence of a freshly prepared RNA extraction buffer (3 ml) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5 % sarcosyl, 0.1 M β-mercaptoethanol and precooled to 4 °C (method 'A'). In later experiments frozen tissue was ground to powder using a pestle and mortar precooled with liquid nitrogen, homogenised with the extraction buffer until dissolved and stored at -70 °C for 1 h (method 'B'). Using this buffer, protein was removed by addition of an equal volume of a 1:1 solution of phenol/chloroform, followed by vigorous vortexing for 3 min and centrifugation at 1000 g for 7 min at 4°C. A further extraction was performed on the upper aqueous layer to ensure most proteins (including RNases) had been removed. RNA was precipitated by addition of 0.1 volume of 3M sodium acetate and an equal volume of propan-2-ol (HPLC grade) to the aqueous layer obtained from the second phenol/chloroform extraction. The mixture was vortexed vigorously, placed at -70 °C for 1 h and pelleted by centrifugation (30,000 g, 75 min, 4 °C). The pellet was washed with 75 % ethanol and air dried for 10 min before resupension in 30 µl of DEPC-treated water.

Later extractions of total RNA from lung were performed using RNAsol B (ams Biotechnology). Frozen tissue (50 mg) was ground to powder as above, dissolved in 1ml RNAsol B as recommended by the manufacturer and stored at -70°C (method 'C'). Where cell cultures or isolates were used, the cells were extracted with 1 ml RNAsol B

per 5 x 10^6 cells. The samples were thawed and RNA extracted by addition of 100 µl chloroform and centrifugation at 12,500 g for 15 min at 4 °C. An equal volume of isopropanol was added to the supernatant to precipitate the RNA on ice for 15 min which was then pelleted by further centrifugation at 12,500 g for 15 min. The pellet was washed in 70 % ethanol, dried and resuspended in 50 µl DEPC-treated water. Unless otherwise mentioned, all results presented in this thesis refer to extractions of total RNA using RNAsol B.

Where possible, the quantity of RNA in individual samples was assessed by absorbance at OD_{260}, and calculated by:

Amount of RNA (µg) = A_{260} x dilution factor x volume of solution (ml) x 40*

* An OD_{260}=1 indicates a solution of approximately 40 µg of RNA per ml

(OD_{280} assesses the amount of protein within the sample).

2.1.5.2. Separation of total RNA by electrophoresis

All total RNA preparations were separated using the following method as outlined by Jordan *et al.* 1995. For each sample total RNA (5-10 µg) was denatured by addition of 30 µl RNA sample buffer (see Appendix 1) and heating to 80 °C for 30 min. RNA was separated by electrophoresis through a denaturing agarose gel containing x1 MOPS and 6.5 % formaldehyde, at 100 V for 90 min, using x1 MOPS as running buffer. The gels were visualised and photographed under UV light (Polaroid film 55) to show differences in loading of total RNA between samples by the ribosomal RNA 18S and 28S bands. These bands also serve as 2 kb and 5.4 kb size markers respectively.

2.1.5.3. Transfer and probing of mRNA

Establishing hybridisation temperatures and buffers for the cDNA probes

Since a number of buffers are used in conjunction with DIG-labelled probes (Vignaud *et al.* 1994, Sambrook *et al.* 1989) the initial optimisation of hybridisation conditions was crucial. Total RNA was extracted using RNAsol B from $5x10^6$ guinea pig lung fibroblast JH4-Cl1 cells harvested at 24 h following stimulation with LPS-stimulated MØ supernatant. The RNA was divided between 4 gel lanes and separated by electrophoresis as above. RNA was then blotted from the gel onto nitrocellulose membrane overnight using 20x SSC (diluted from a x20 stock containing 3 M NaCl, and 0.3 M trisodium citrate pH7.0) and stabilised by baking the membrane at 120 °C for 20 min. Strips were cut from the membrane (1 lane/strip) and probed using a DIG-labelled cDNA for gpIL-8 in the presence of differing buffers and temperatures as shown below.

Buffer	Constituents	Recommended hybridisation temperature
A.'Standard buffer'	5x SSC, 0.1% sarcosyl, 0.2% SDS, 1% blocking buffer DEPC water	42 °C
B.'High formamide standard buffer'	5x SSC, 0.1% sarcosyl, 0.02% SDS, 1% blocking buffer 50% formamide DEPC water	68 °C
C.'High SDS buffer'	5x SSC, 0.1 % sarcosyl, 7% SDS, 2% blocking reagent, 50% formamide 50 mM sodium phosphate buffer, pH 7	42 °C-50 °C

See Appendix I for SSC and blocking buffer constituents.

Table 2.1. Hybridisation buffers used to optimise conditions for the use of DIG-labelled cDNA probes in Northern blot analysis

Transfer and detection of probe

For all experiments RNA was blotted from the gel onto nitrocellulose membrane overnight using 20x SSC and stabilised by baking the membrane at 120 °C for 20 min. Unless otherwise indicated all membranes were first prehybridised at 50 °C using the high SDS hybridisation buffer (10 ml/50 cm^2) for 1 h. This was replaced by fresh buffer (1.25ml/50 cm^2) containing 10 ng/ml denatured DIG-labelled DNA probe (denaturation by heating probe at 95 °C for 5 min) and hybridised at 50 °C overnight. Non-specific

bound probe was removed by washing the membrane twice at low stringency in 2x SSC containing 0.1 % SDS for 5 min and twice at high stringency in 0.1x SSC/0.1 % SDS for 5 min. After a brief rinse in wash buffer (0.3 % Tween-20 in maleic acid buffer), the membrane was blocked with 1 % blocking buffer (10 % blocking stock diluted in maleic acid buffer) for 30 min, before detection of the bound probe by incubation with anti-DIG-AP Fab fragments (0.075U/ml) in 1 % blocking buffer for 1 h. Excess antibody was removed by wash buffer (3x10 min), and the membrane immersed briefly in substrate buffer before addition of CSPD chemiluminescence substrate in buffer giving a final concentration of 0.25 mM and allowing 500 µl/50 cm^2. The membrane was sealed in polythene and incubated at 37°C for 30 min. The membrane was exposed to Kodak X-OMAT AR5 film (Sigma, Poole, UK) for 2 h, and developed.

2.1.6. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

2.1.6.1. Extraction of poly (A) RNA

Poly (A) RNA was extracted using QuickPrep *Micro* mRNA Purification Kit in accordance with the manufacturer's instructions. Briefly, 100 mg of frozen guinea pig lung was solubilised in 0.4 ml extraction buffer containing guanidinium thiocyanate and 0.8 ml elution buffer. Cell debris was removed by centrifugation (12,500 g for 1 min) and the supernatant mixed with oligo(dT)-cellulose for 3 min. Following another centrifugation as above, the pellet was washed with high salt buffer (x5) followed by low salt buffer (x2), transferred to a microspin column from which mRNA was eluted with 200 µl elution buffer.

2.1.6.2. Reverse-transcription reaction

Poly (A) RNA was reverse transcribed to generate cDNA. Initially, 5 µl of the QuickPrep column eluate was diluted to a final volume of 12.5 µl and denatured at 75 °C for 3 min. The solution was cooled at 4 °C before addition of 7.5 µl of RT 'reaction mixture' containing sufficient reagents for optimal concentration in 20 µl; random hexamers (Pd $(N)_6$) at final concentration of 1 µM, RT buffer x1, dNTPs each at 0.5 mM, RNasin at 1 U/µl and M-MLV RTase at 10 U/µl. Reverse transcription was performed at 42 °C for 60 min, followed by a denaturation at 95 °C for 2 min. The product was cooled on ice and underwent PCR immediately.

2.1.6.3. PCR of RT product

The cDNAs for gpRANTES and gpMIP-1 were amplified in 35 cycle PCR reactions using a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer, CA, USA) based on a method by *Huang et al* (1994). Each lung sample cDNA template was diluted and transferred to separate PCR tubes such that there were 3 tubes per sample containing 3 µl, 1.5 µl and 0.3 µl of RT product with volumes made up to 15 µl with DEPC-treated water. To each tube, 8 µl of reaction mixture was added containing suitable amounts of constituents to deliver final concentrations of; x1 PCR buffer, 200 mM each of dATP, dCTP. dGTP and dTTP, 1 mM each of appropriate forward and reverse primers (table 2.2) and 0.5U Taq polymerase. Primer sequences were selected so as to minimise primer dimerisation between pairs and with the selected house-keeping gene (β-actin) sequence which served as a internal standard for semi-quantitative analysis (see table 2.2). As well as RANTES and MCP-1, attempts were made to amplify eotaxin reverse

transcripts using two primer sets; *reverse* 5' CAT CTC AAG CAC GAT GAT TAC 3' with *forward* primer 5' ACA CTG CAC CAT GAA AGT CTC 3', and *reverse* 5' TCC TGA ACC CAC TTC TTC TTG 3' with *forward* 5' CTG CAC CAT GAA AGT CTC CAC 3'. Where co-amplification of both chemokine and β-actin sequences was not possible, the PCR for each primer pair was conducted separately. Cycle conditions were set as before except the annealing temperature of the reaction was set at 55 °C over 35 cycles. The PCR product was stored in aliquots at -20 °C.

To ensure that amplified DNA originated from reverse transcription and was not genomic DNA carried over from the original mRNA extraction, an equivalent volume of non-reverse transcribed mRNA was placed directly into the PCR reaction. The reaction mixture was analysed by electrophoresis, following 35 PCR cycles as described previously.

Protein	Primer Sequence 5'-3' R=Reverse, F=Forward	Product length	Annealing temperature
gpRANTES	**R** TAT GCC TCA GAT ACC ACT CC **F** GTA ATA GAA TCC ATG CCT CC	223 base pairs	55.3 °C
gpEotaxin	**R** CAT CTC AAG CAC GAT GAT TAC **F** ACA CTG CAC CAT GAA AGT CTC	318 base pairs	56.6 °C
gpMCP-1	**R** CCC AAG CTT CAC TCT ACT TGT AGA ATT TGGA A **F** CAG CCG GAT GGA GTT AAT AC	470 base pairs	57.3 °C
gpMIP-1α	**R** AAT CTT TGA TTC CTC CCA GG **F** GCT GCT TCA ACT ATG CCT CC	208 base pairs	57.0 °C
gpIL-8	**R** TTC ACA CCA CAC CTT TCC AC **F** GCC CTT GAT CTT AAT TTT GCT C	512 base pairs	55.7 °C
β-actin	**R**. CTT TTC CAG CCT TCC TTC **F**. GCA GTA ATC TCC TTC TGC ATC	176 base pairs	55.1 °C

Table 2.2. Primer sequences for PCR amplification. Primers were designed using the 'Primer' programme of the GCG package (Daresbury Laboratory, Cheshire, UK)

2.1.7. Analysis of RANTES protein in whole guinea pig lung using Western blotting

2.1.7.1. Assessment of anti-RANTES monoclonal and polyclonal antibodies

A panel of 11 anti-human RANTES monoclonal antibodies was supplied by Dr. T Wells (Glaxo Wellcome, Geneva, Switzerland). Anti-guinea pig RANTES antisera was generated in rabbits by Dr. M Watson (Bath). All further characterisation of the antibodies was carried out by myself at Bath University.

Assessment by ELISA. Monoclonal antibodies were initially characterised for their ability to recognise recombinant guinea pig RANTES protein by ELISA using a method adapted from Harlow (1992). Serial dilutions of the chemokine (50 µl) in sodium bicarbonate buffer containing 20 mM Na_2CO_3 and 70 mM $NaHCO_3$ at pH 9.6, were used to coat Nunc MaxiSorp plates (Gibco BRL) overnight at 4 °C. Plates were then washed with TBS containing 0.05 % Tween 20 (wash buffer) and wells incubated with dilutions of each monoclonal antibody for 2 h at 37 °C. Dilutions were made in the wash buffer containing 2 % FCS and a final volume of 50 µl of antibody was added to the wells. Following the incubatory period, excess antibody was removed by three washing steps. Bound antibody was detected by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody (100 µl/well using a 1/15,000 dilution) for 90 min at 37°C, washing the plate with TBS twice and addition of substrate *p*-nitrophenylphosphatase. The substrate was purchased as Sigma 104 Phosphatase tablets and diluted in 1 M tris pH 9.7 at 1 µg/ml, and 100 µl added to each well. Colour development was measured after 30 min at 405 nm using a Titertek plate reader (ICN Biomedicals, Thame, UK).

Dot blot analysis. Selected monoclonals and polyclonal antisera were also tested by dot blot analysis. Nanogram quantities of chemokine were dotted as 1 µl onto multiple nitrocellulose membrane strips and allowed to dry. The strips were blocked using a non-fat milk-based blocking buffer (5 % Marvel in TBS) overnight before incubation with different primary antibodies. Primary antibodies were diluted in TBS containing 1 % Marvel. After 3 h the membranes were washed for 10 min in TBS, three times for 10 min in TBS with 0.05 % NP-40 detergent, and finally for 10 min in TBS again. The membrane strips were then incubated with 1/10,000 dilution of alkaline phosphatase-conjugated secondary antibody (goat anti-mouse for monoclonals, goat anti-rabbit antibodies for polyclonals). Excess secondary antibody was removed by immersion of the membranes in TBS containing 0.05 % NP-40, threee times for 10 min and twice in TBS, and bound antibody detected using Enhanced Chemiluminescence (ECL) according to the manufacturer's instructions.

Western blot analysis of recombinant RANTES protein. Antibodies were also assessed for their ability to identify RANTES under denaturing conditions. Full methodology for Western blot analysis is described in the section below. Briefly, recombinant hRANTES and gpRANTES were run in multiple lanes of a 15 % SDS-PAGE gel using a Bio-Rad Mini Protean gel system. Proteins were transferred onto nitrocellulose, which was cut into strips such that each strip contained a sample of both rhRANTES and gpRANTES. Each membrane was blocked for non-specific binding and incubated in 1 µg/ml of a different monoclonal antibody for 3 h. Bound antibody was detected using alkaline phosphatase-conjugated goat anti-mouse secondary antibody and ECL as described above.

2.1.7.2. Extraction and separation of protein from guinea pig lung

Frozen whole lung tissue (approximately 100 mg) was homogenised in 0.01 % triton X-100 containing 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mg/ml EDTA. The homogenate was centrifuged at 13,000g for 2 min and supernatant stored at -20 °C. An assessment of the total amount of protein in each sample was obtained by comparing serial dilutions of each sample with a standard curve of known amounts of BSA using Bio-Rad Protein Assay, based on the Bradford dye-binding procedure (Bradford, 1976). Briefly, 10 μl of sample or standard was mixed with 150 μl of the reagent and samples were read at 595 nm using a Titertek plate reader (ICN Biomedicals, Thame, UK). The concentration of triton-X (0.01 %) was compatible with this assay.

For each lung sample, approximately 2 mg of total protein was separated by SDS-PAGE on a 15 % running polyacrylamide gel under denaturing conditions (see Appendix 1 for details of gel composition) using a Bio-Rad Mini Protean system. The samples were run at 50 V for 15 min through the stacking gel (4 %) and at 150 V for 1.5 h through the running gel. Following electrophoresis the gels were allowed to soak in transfer buffer for 20 min before transfer of the protein onto nitrocellulose membrane (Bio-Rad, Hemmel Hempstead, UK) using 250 mA at 90 V for 40 min according to the manufacturer's instructions. Membranes were blocked with blocking buffer and probed with optimal concentrations of primary antibodies which were detected using the respective secondary antibody and ECL as described above in section 2.1.7.1.

Where required, some gels were not transferred but stained with a solution of 0.2 % Bio-Rad Coomassie Blue Reagent-250 containing 45 % methanol and 9 % acetic acid for 5 h and then destained in a 10 % solution of methanol with 10 % acetic acid until the protein bands were clearly visible.

2.2. PURIFICATION AND CHARACTERISATION OF RECOMBINANT GUINEA PIG RANTES PROTEIN

2.2.1. Cloning of gpRANTES

Full length gpRANTES cDNA was cloned by T. Yoshimura (NCI-FCRDC, MD, USA) from a guinea pig spleen cell library while screening for gpMCP-1 (Yoshimura 1993). Briefly, poly (A) RNA was prepared from guinea pig splenocytes stimulated with concanavalin A (5 μg/ml, 4 h). A modification of the Gubler and Hoffman method (Gubler & Hoffman 1983) was used to synthesise cDNA. This was used to prepare a library in the ZAP II vector which was probed with a ^{32}P-labelled oligonucleotide probe for MCP-1. Positive phagemids were rescued with helper phage and sequenced by the dideoxynucleotide chain termination method in accordance with the manufacturer's instructions (Stratagen, La Jolla, US).

2.2.2. Gene expression

Successful expression of gpRANTES in *E. coli* was achieved by mutation of a human RANTES construct previously described (Proudfoot *et al.* 1995). The original construct contained the coding sequence of mature human RANTES with the addition of a N terminal hexapeptide ending in arginine (Arg) at the 5' end. Base substitutions were made by site directed mutagenesis to achieve amino acid changes at the six positions giving rise to the differences in primary sequence between guinea pig and human RANTES. Therefore, the protein produced was identical to the predicted gpRANTES amino acid sequence. The constructs were subcloned into the expression vector pT7-7 and transformed into *E. coli* strain BL21 induced by addition of isopropylthiogalactoside to the medium. This work was carried out by B. Allet (Geneva Biomedical Research

Institute, Glaxo Wellcome, Switzerland). All subsequent procedures were carried out by myself at Glaxo IMB, Geneva , under the supervision of Dr. A. Proudfoot.

2.2.3. Protein Purification

2.2.3.1. Extraction from inclusion body

Guinea pig RANTES was purified essentially as described for human RANTES by Proudfoot and co-workers (Proudfoot *et al.* 1995). For the purposes of comparison and familiarisation with the purification techniques, rhRANTES was also purified. Differences in the purification of the proteins are outlined below, where necessary.

Guinea pig RANTES protein was extracted from transformed *E. coli* (40g) by four rounds of homogenisation, sonication and French Press extraction in lysis buffer (Tris-HCl 50 mM pH 7.6, DTT 1 mM, PMSF 1 mM, DNase 20 mg/ml, $MgCl_2$ 16 mM) allowing the homogenate to cool at 4 °C for 5 min between each round. The extract was centrifuged at 10,000g for 60 min, and the hexapeptide-gpRANTES fusion protein identified as a component of the inclusion body by SDS-PAGE. The cell pellet was solubilised in 100 ml of 0.1 M Tris-HCl buffer (pH 8) containing 6 M guanidine HCl and 1 mM DTT and denatured by heating to 60 °C for 30 min before initial separation by size exclusion gel filtration on a Sephacryl HR S200 column (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. Briefly, the protein was loaded onto the column and eluted with the same buffer at a rate of 8 ml/min in 20 ml fractions. Aliquots (1 ml) of all fractions were dialysed against 6 M urea to allow analysis by SDS-PAGE and those containing protein between 5-20 kDa were pooled.

2.2.3.2. Renaturation and further purification

The concentration of hexapeptide-gpRANTES protein in these fractions was determined and the solution diluted with the same Tris/guanidine/DTT buffer to achieve a final protein concentration of 1 mg/ml. The protein was renatured by a 20-fold dilution into 0.1 M Tris-HCl (pH 8.5) buffer containing 1 mM oxidized and 0.1 mM reduced glutathione, at a rate of 1.5 ml/min with constant stirring at 4 °C overnight. Non-renatured protein was removed by centrifugation, and the renatured protein was then concentrated using cation exchange chromatography. Initially, the solution was adjusted with to pH 4.5 with NaOH, and diluted with distilled water to achieve a conductivity of 10 mS before applying to a HiLoad SP 26/10 column (Pharmacia) equilibrated with 50 mM sodium acetate, pH 4.5. Absorbed protein was eluted with a linear 0-2 M NaCl gradient in the same buffer over 4 h, at a rate of 8 ml/min and collected as 20 ml fractions. SDS-PAGE identified fractions containing the hexpeptide-RANTES protein which were pooled, and the protein concentration determined by absorbence at OD_{280}. The solution was frozen using a methanol-dry ice bath and the protein lyophilised.

2.2.3.3. Removal of hexapeptide sequence and separation of digestion products

The protein (1 mg/ml) was solubilised in 50 mM Tris-HCl (pH 8) and the hexapeptide leader sequence was removed from the RANTES protein by incubation with Endoproteinase Arg C at 1:300 (w/w enzyme:substrate) for 6 h at 37 °C. This length of incubation was optimal and determined by RP-HPLC analysis of the digestion at 1 h intervals from 1-8 h.

Products resulting from the cleavage of both hexapeptide-gpRANTES and hexapeptide-hRANTES underwent initial separation by cation exchange chromatography under the

same conditions as described above with the addition of 6 M urea in the buffers and eluting the protein with a 0-50% gradient of NaCl over 3 h (5 ml/min in 10 ml fractions). This effectively separated the products of the hexapeptide-human RANTES digestion. However, this process only partially separated the hexapeptide-gpRANTES digestion. Complete recovery of cleaved gpRANTES was achieved by RP-HPLC. Protein (1 μg in 2 ml) was first acidified with TFA such that the final concentration of acid was 0.1% before injection onto a VarioPrep Nucleosil 300-7 C8 column (250 x 10 mm, all HPLC columns from Macherey-Nagel, Düren, Germany), equilibrated with 0.1% TFA in water. Protein was eluted at a rate of 4 ml/min with a gradient of buffer A (0.1% TFA) and buffer B (90% acetonitrile with 0.1%TFA in water) using 25-50% buffer B over 25 min. All buffers were filter sterilised. The peak containing cleaved gpRANTES was identified and the protein lyophilised in preparation for biological characterisation.

2.2.3.4. Analytical methods.

Protein purification and purity were followed by SDS-PAGE using 4-20 % acrylamide precast mini-gels (Novex) following manufacturers instructions and the gels were stained with Coomassie Brilliant Blue R250. All samples were denatured by mixing with an equal volume of x2 sample buffer and heating to 95 °C for 5 min. RP-HPLC was also employed using a Beckman System Gold with an Aquapore RP 300-7 C8 (220 x 2.1 mm, Macherey-Nagel) analytical column at a flow rate of 1 ml/min using the same sample preparation and column conditions as described above. The purified proteins were quantified by the extinction coefficients of $A_{1cm}^{0.1\%}$ = 2.1 and 1.6 at OD_{280} for the hexapeptide-gpRANTES fusion and gpRANTES respectively, calculated from the

predicted amino acid sequences. The amino terminus was verified by Edman degradation using a model 477A (Applied Systems) pulsed liquid phase sequencer. The amino acid composition was verified by overnight hydrolysis at 105 °C in 6 M HCl and quantified using a Beckmann 6300 amino acid analyser with a SICA integrator

Bioactivity of the gpRANTES purification product was monitored by elevation of $[Ca^{2+}]_i$ using the human monocytic cell line THP-1. The cells were cultured in suspension in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM (L)-glutamine, 0.01 M HEPES and 0.005% gentamycin and passaged to maintain an optimal density of 5-8x10^5/ml. The cells were incubated with 2.5 μM fura-2AM for 30 min at 37 °C, washed three times in buffer and resupended at 2x10^6 cells/ml. Simultaneous measurements were made at an excitation of 340/380 nm and emission of 510 nm using an LS50 Perkin Elmer spectrofluorimeter. An increase in the 340/380 ratio was taken to indicate bioactivity.

2.2.4. Attempts to identify RANTES as a product of guinea pig platelets

In an attempt to identify a native source of gpRANTES for the purposes of sequencing, platelet lysates were analysed for the possible presence of the protein using a method adapted method from Kameyoshi *et al.* (1988) who isolated this chemokine from human platelets.

Isolation of platelets. Approximately 30 ml of blood was collected from two Sagatal-anaethetised Dunkin Hartley guinea pigs (800-900 g) by cardiac puncture into 3 ml of 2.5 % sodium citrate containing 1.5 % citric acid and 2 % glucose. Platelet rich plasma was prepared by centrifugation of the citrated whole blood at 350 g for 15 min. The

upper layer was collected and centrifuged further at 1000 g for 10 min to pellet the platelets. They were then resuspended at $2x10^8$/ml platelets in Hepes-buffered Tyrodes solution (pH7.4) containing 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose and 0.25 % BSA.

Thrombin-stimulated platelet aggregation. Platelet activation was monitored by a aggregation method using a Chronolog dual aggregometer as described by Born (1962). This method detects changes in light transmission through the platelet suspension as a result of an aggregation response to an agonist. Platelet shape change induces a decrease in light transmission whilst aggregation causes an increase. Light transmission (0 %) was calibrated by assessing the transmission through $2x10^8$ platelets per ml whilst 100 % transmission was assumed to be represented by transmission through a ten fold dilution of the platelet suspension ($2x10^7$/ml). In order to assess aggregation in response to an agonist, 0.5 ml of platelet suspension ($2x10^8$/ml) was stirred at 37 °C for 2 min. Calcium was added to a final concentration of 1 mM at t=0 min and thrombin (2U/ml) at t=2 min whilst continually stirring at 900 rpm at 37°C. Platelets were removed by centrifugation (1000 g for 5 min) at 30 min after the addition of thrombin (as described for human platelets by Kameyoshi *et al.* 1988) and the supernatant stored at -80 °C.

Assessment of activity of thrombin-stimulated platelet supernatant (TSPS). Thrombin-stimulated platelet supernatant (TSPS) was assayed for its ability to increase $[Ca^{2+}]_i$ in guinea pig peritoneal macrophages using the same methods as described in sections 2.2.5.1-2 of this thesis. Following macrophage equilibration with 1mM Ca^{2+}, various volumes of TSPS were used as a potential agonist. The effect of the PAF antagonist WEB 2086, on the changes in $[Ca^{2+}]_i$, was assessed by addition of a saturating dose (1uM) to the platelet suspension at 1 min prior to addition of TSPS.

2.2.5. In vitro biological characterisation

2.2.5.1. Cell isolation

Guinea pig peritoneal eosinophils were generated by twice-weekly intraperitoneal injections of horse serum over 4 weeks using 400-500 g Dunkin-Hartley guinea pigs (Harlan, UK) with an injection the day before harvesting (Litt 1960). Macrophages were elicited by one 5 ml intraperitoneal injection of thioglycollate (0.05 % w/v) and the cells were harvested after 3 days. Guinea pigs were killed by CO_2 asphyxiation, a small mid-line incision made in the abdomen, and the peritoneal cavity lavaged with 2x30 ml HBSS pH 7.4 containing 1 mM EDTA. The cells were washed with HBSS containing 0.1% BSA (<0.1 ng endotoxin/mg) and resuspended in Percoll (1.070 g/L) containing 1% BSA. The leukocyte populations were separated by centrifugation (1500 g for 25 min at 4 °C) on a discontinuous isotonic Percoll gradient (Shute *et al.* 1990) through densities of 1.070, 1.080, 1.085, 1.090, 1.100 g/L. Eosinophils and macrophages separated at the 1.095-1.100 and 1.080-1.090 interfaces respectively. The cell containing fractions were carefully removed and washed three times in HBSS containing 0.1% BSA. In each case the cell purity obtained was >96% and the major contaminant cell type was monocytic with some neutrophils in the case of the eosinophil preparations, and neutrophilic in the case of the macrophage preparations.

Human eosinophils were isolated from peripheral whole blood using a negative selection technique adapted from Hansel (1989). Citrated blood (100 ml of peripheral blood containing 0.38 % of sodium citrate) was pelleted by centrifugation at 1865 g for 6 min at 4 °C and the plasma removed. The red blood pellet was diluted to the original volume with RPMI 1640, layered onto Lymphoprep (2:1 v/v) and spun at 400 g for 30 min at 20 °C. Peripheral blood mononuclear cells were removed and the remaining

leukocyte/erythrocyte sediment was mixed with dextran T500 and the volume replenished to 150 ml with saline such that the dextran was at a final concentration of 1%. The suspension was allowed to sediment for 30 min after which time the leukocyte-rich supernatant was spun (350 g, for 10 min at 4 °C). Remaining red blood cells were removed from the resulting cell pellet by hypotonic lysis with cold H_2O and washing with RPMI/EDTA and the granulocytes collected by centrifugation (200 g for 10 min at 4° C). Eosinophils were isolated by negative selection using anti-CD16 magnetic particles and a MACS separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, the cells were mixed with anti-CD16 magnetic particles for 30 min at 4 °C ($2x10^8$ cells/100 ml beads). The cells were applied to the MACS column and the eosinophils eluted with 8 column volumes of RPMI 1640. The resulting cell purity was >96% and the contaminating cells were neutrophils and monocytes.

Guinea pig leukocytes were isolated from whole blood obtained from animals injected with recombinant human IL-5 (18.3 pmol/kg) and bled after 1 h, according to methodology by Collins *et al.*1995. Blood was collected into sodium citrate (0.38 % w/v final) and sedimented with 1 % dextran as described for human eosinophils. The upper layer was removed and cells pelleted by centrifugation at 350g for 10 min. Attempts were made to separate these cells using a discontinuous Percoll gradient as described for guinea pig peritoneal eosinophils. Cells were then washed with HBSS containing 0.1 % BSA twice before resuspension in HBSS/BSA with 1 mM Ca^{2+} and Mg^{2+}.

2.2.5.2. Measurement of intracellular calcium

Cells were suspended in 5 ml of HBSS/0.1% BSA containing 2.5 μM fura2-AM and incubated at 37 °C for 30 min. The cells were washed three times in HBSS/BSA and resuspended in HBSS/BSA containing 1 mM Mg^{2+} and 0.1 mM Ca^{2+} at $1x10^6$ cell/ml. Prior to each experimental run, extracellular Ca^{2+} was restored to 1 mM. Fluorescence was monitored in all cell aliquots at excitation wavelengths 340 and 380nm and emission at 510 nm with constant stirring at 37 °C in a Deltascan spectrofluorimeter (PTI inc., Surrey, UK). Baseline fluorescence was monitored for 20 sec before addition of agonists (10 μl). Signals were calibrated by monitoring the fluorescence change in a cell suspension after addition of 12.5 mM digoxigenin followed by 40 mM NaOH and 4 mM EGTA 30 sec later, to obtain values of R_{min} and R_{max}. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence using the excitation wavelengths according to Grynkeiwcz (1985):

$$[Ca^{2+}]_i = Kd \times \frac{(R-R_{min})}{(R_{max}-R)} \frac{Sf_2}{Sb_2}$$

Kd = the dissociation constant for fura-2 at 37 °C is $2.24x10^{-7}$ M

R = Ca^{2+} bound/Ca^{2+} free flourescence

R_{min} = fluorescence ratio with zero Ca^{2+},

R_{max} = fluorescence ratio under saturated calcium conditions

Sf_2/Sb_2=ratio of fluorescence values for Ca^{2+} bound/Ca^{2+} free indicator measured at the wavelength used to monitor Ca^{2+} free indicator (i.e. 380 nm).

2.2.5.3. Leukocyte chemotaxis

Cells were resuspended at $2x10^6$/ml in HBSS containing 1 mM Ca^{2+}/Mg^{2+} and 0.1 % BSA. Chemokine dilutions at 1-100 nM were prepared by dilution in the same buffer. Chemokines, C5a as a positive control or buffer as a negative control, were added to the lower wells of 48 well microchemotaxis chambers (Neuro Probe, Cabin John, US) and covered with a polycarbonate filter with 5 μm pores for eosinophil experiments and 8 μm pores for macrophages. For each experiment, 4 replicates of each sample dilution were performed. Cells were added to the upper chamber and allowed to migrate for 2 h at 37 °C. The membrane was removed after incubation, washed, fixed and stained to allow visualisation of migrated cells. Results were expressed as mean ± SEM per high power field (all cells in the field of view using a magnification of x400) using cells preparations from *n*=3 or 4 different animals. In some eosinophil experiments, IL-5 was added together with chemotactic agents into the lower wells and the cells allowed to migrate as described.

2.2.5.4. Measurement of hydrogen peroxide generation.

Hydrogen peroxide generation from RANTES-stimulated cells was determined by oxidation of the fluorescent reagent scopoletin to a non-fluorescent product at 37 °C as previously described by Nathan (1987). Ninety-six well flat-bottomed flexiplates were precoated with 150 μl of a 3 mg/ml laminin solution for 1 h at 37 °C. The plate was washed with HBSS and cells resupended in HBSS containing 1.5 mM Ca^{2+} and Mg^{2+} were added to each well (100 μl at $2x10^6$ cells/ml). The following reagents were then added per well; 50 μl HBSS, 10 μl 'reaction mixture' containing scopoletin (3.6 nmol; final), horse radish peroxidase (10 μg), sodium azide (150 nmol) and Ca^{2+}/Mg^{2+} (20

nmol). The plate was incubated at 37 °C for 15 min prior to addition of varying amounts of test agonists in 10 µl. Hydrogen peroxide generation was monitored by a decrease in fluorescence using an excitation wavelength of 355 nm and emission of 460 nm detected by a Titertek II plate reader (ICN Biomedicals, Thame, UK). A time zero reading was taken immediately after addition of test reagents and at 10 min intervals thereafter.

Calibration of hydrogen peroxide production. A standard curve of scopoletin fluorescence in the presence of known quantities (1-6 nmols) of hydrogen peroxide was established by addition of 150 µl HBSS, 10 µl reaction mixture and 10 µl of the respective H_2O_2 standard to blank wells. Each H_2O_2 standard and test reagents was performed in triplicate wells. The fluorescence readings from test reagent wells were converted to nmol of H_2O_2 from the fitted standard curve.

The spread sheet Excel (version 5) was used to manipulate the data and calculate the H_2O_2 generation from changes in fluorescence.

2.2.6. Assessment of chemoattractant activity of RANTES in vivo

2.2.6.1. Tracheal instillation of gpRANTES

Dunkin-Hartley guinea pigs (350-400 g either sex) were anaesthetised with a mixture of ketamine (40 mg/kg) and xylazine (5 mg/kg), placed prone and tracheal instillation performed with the aid of a laryngoscope. gpRANTES (0.1-10 μg) was instilled as 50 μl with saline/0.1% BSA as a vehicle using a Gilson pipette fitted with a gel-loading tip. The animals were killed at 6 h, 24 h or 48 h post treatment with an overdose of pentobarbitone (Euthatal). The trachea was cannulated to allow attachment of a syringe containing 10 ml of saline with 0.1% BSA and 1 mM EDTA. This volume was gradually emptied into the lung so as to minimise tissue damage, and carefully syringed back after 10 sec. For each lung this lavage process was repeated three more times with fresh buffer and the lavage fluid pooled. Total cell counts were determined and BAL cell cytospins prepared using a Shandon cytospin (Shandon Scientific Ltd, Runcorn, UK), stained with 'Diff-Quik', and differential cell counts performed. All results were expressed as mean±SEM cell number of *n* animals. Cell numbers from RANTES-instilled animals were compared to those from vehicle (saline with 0.1 % BSA) instilled animals at the appropriate time point.

2.2.6.2. Intradermal injection of gpRANTES into naïve guinea pig skin sites

A number of methods were employed to investigate the effect of RANTES on eosinophil accumulation in the guinea pig skin.

Accumulation of ^{111}In-labelled peritoneal eosinophils. Accumulation of eosinophils in the guinea pig skin was monitored using a method adapted from Faccioli *et al.* (1991). Peritoneal eosinophils were generated by repeated horse serum injections using 600-

700g 'donor' Dunkin Hartley guinea pigs and isolated as described above. The cells were labelled with $^{111}InCl_3$ (100 μCi in 100 μl) chelated with 2-mercaptopyridine-*N*-oxide (40 μg in 0.1 ml of 50 mM PBS pH 7.4) for 15 min at room temperature. Labelled cells were washed twice and resuspended at a density of 10^7 eosinophils /ml in Hank's buffer pH 7.3 containing 0.1 % BSA.

'Recipient' guinea pigs (350-400 g) were sedated with Hypnorm (0.15 ml i.m.) and their dorsal skin shaved and skin sites marked. Each animal received $5x10^6$ labelled cells mixed with 2.5 μCi of ^{125}I-albumin via the ear vein. Various mediators were injected i.d. as 100 μl volumes into separate sites of the dorsal skin. Saline was used as a vehicle control whilst guinea pig zymosan activated plasma (30 % in saline) was used as a positive control for eosinophil accumulation and histamine for plasma leakage. Each animal received a duplicate of each treatment according to a randomised balanced site injection plan. After 2 h the animals were killed with an overdose of Euthatal (sodium barbitone) and a cardiac blood sample was taken immediately and plasma separated from cells by brief centrifugation. The dorsal skin was removed and skin sites were punched out with a 17 mm diameter punch and samples were counted together with plasma samples using an automatic gamma counter (Wallac, Milton Keynes, UK). Samples were also counted for ^{125}I and corrections were made for cross-talk between the two isotopes.

Total ^{111}In counts for a known number of eosinophils enabled calculation of ^{111}In counts per eosinophil and this was used to determine ^{111}In eosinophils per skin site. Plasma leakage was expressed as μl of plasma by dividing the skin sample ^{125}I counts by ^{125}I counts per 1 μl of plasma.

Accumulation of blood-derived cells. Dunkin Hartley guinea pigs (350-400 g) were anaesthetised with Hypnorm (0.15 ml i.m.) and after 5 min the dorsal skin shaved. gpRANTES was injected i.d. as 100 µl volumes into separate sites of the dorsal skin, to give final amounts of either 10^{-10} or 10^{-11} mol/site. Other sites were injected with 100 µl saline as a vehicle control or guinea pig zymosan activated plasma (30% in saline) as a positive control and each animal received a duplicate of each treatment following a randomised injection plan. The inflammatory response was assessed at 2 and 24 h (n=3 animals for each time point) by overdosing with sodium pentobarbitone (Euthatal) and removing the skin sites with a 17 mm punch. Samples were fixed in formal buffered saline overnight, embedded in paraffin wax and 8 µm sections cut to show a transverse view through the injected site. Mounted sections were stained by H&E to allow identification of dermal infiltrates.

Preparation of zymosan-activated plasma (ZAP). Zymosan-activated plasma was used as a rich source of guinea pig C5a-des-Arg. Guinea pigs were killed by CO_2 asphyxiation and cardiac puncture performed to remove 10-15 ml of blood into a syringe loaded with heparin allowing 10 IU/ml blood. The supernatant was removed after sequential spinning at 1350 g for 15 min and 1950 g for 15 min. The plasma was incubated with zymosan (5 mg/ml) for 30 min at 37 °C with occasional shaking after which time the zymosan was removed by centrifugation at 3000 g for 15 min. The supernatant was purified using a PD10 Sephadex G-25M column to remove low molecular weight material, according to the manufacturer's instructions (Bio-Rad Labs, Richmond, CA, US), and stored at -20 °C before use.

2.2.6.3. Statistical Analysis

Where appropriate, statistical analysis was performed on *in vivo* data to identify statistical differences between treatments and time-matched controls. Data was subjected to one-way ANOVA followed by post-hoc Dunnett's test to compare a number of treatment groups to one control group, whilst a Student's unpaired *t* test was used to compare single treatment groups to their respective control groups.

Differences in standardised levels of chemokine message relative to those of the house-keeping gene, β-actin, were compared between treatment groups using the Mann Whitney U test.

All statistical analysis was performed using Minitab 10.

CHAPTER 3. RESULTS:

Detection of chemokines in the guinea pig lung

To identify the presence of chemokines in the guinea pig lung, whole tissue was analysed using Northern blot analysis, *in situ* hybridisation and RT-PCR.

3.1. Synthesis and validation of molecular probes

Since molecular probes are not currently commercially available to study chemokine expression in the guinea pig, all investigative tools had to initially be synthesised and validated initially. All this work was carried out by myself at Bath University using chemokine cDNA inserts in the pBluescript SK- plasmid as starting material. These were supplied by T. Yoshimura, NCI-FCRDC, MD, USA.

3.1.1. Synthesis of cDNA probes for Northern blot analysis

DIG-labelled cDNA probes were successfully generated by PCR using the primer sequences selected. PCR cycle conditions were set as: denaturation phase at 95 °C for 15 sec, annealing phase at 55 °C for 15 sec and extension phase at 72 °C for 15 sec. The amplification of 5 μg plasmid DNA over 30 such cycles generated approximately 0.2 μg in 100 μl (2 μg/ml) of DIG-labelled probe for all the chemokines, as assessed by dot blot analysis and comparison to a sample of control DIG-labelled DNA (from Boehringer Mannheim) of known concentration (figure 1). DIG-labelled DNA was detected using anti-DIG-AP Fab fragments and a chemiluminescence substrate and this detection system was also used as for the Northern blot analysis studies. The specificity

of amplification for each chemokine insert was confirmed by the presence of fragments of expected size using electrophoresis based on the selected primer sequences (figure 2).

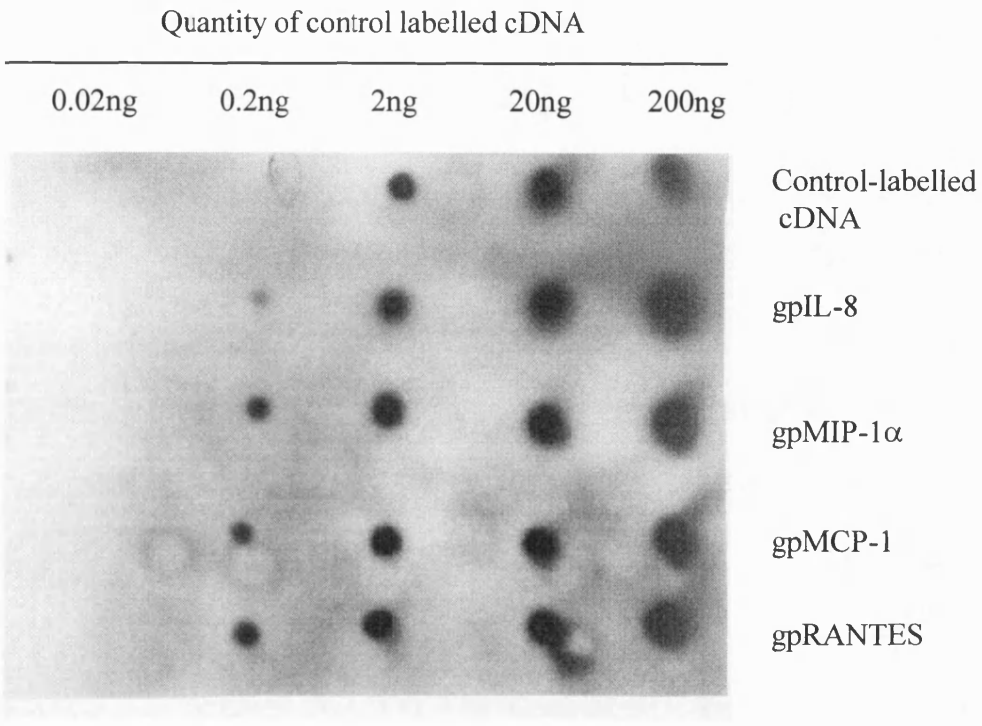


Figure 1. Dot blot analysis of PCR-generated DIG-labelled cDNA probes for guinea pig chemokines. Ten-fold serial dilutions of probes were dotted as 1 µl onto nylon membrane. DIG-labelled DNA was detected using anti-DIG-AP Fab fragments and chemiluminescence substrate. DIG-labelled probe was assessed by comparison with a labelled DNA sample of known concentration (Boehringer Mannheim) as shown. DIG-labelled probes were prepared and quantified on three separate occassions. Each preparation yielded similar quantities of probe as shown above.

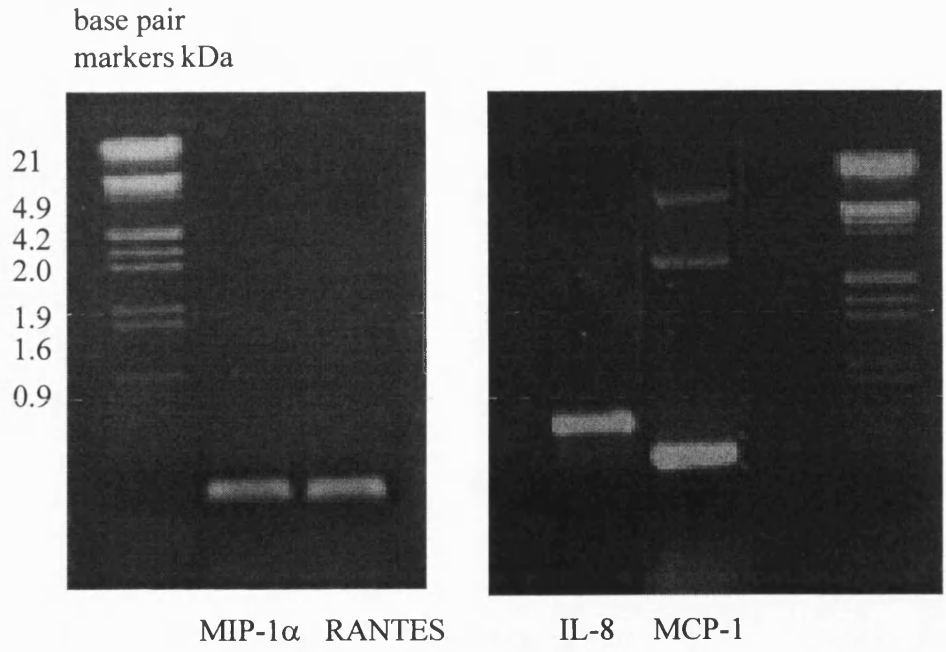


Figure 2. Separation of PCR products following amplification of chemokine cDNA sequences. cDNA were subcloned into pBluescript SK- plasmid and amplified using selected primer sequences to generate DIG-labelled cDNA probes. The main products correspond with the predicted fragment size based on the primer pairs selected; gpMIP-1α; 208 base pairs, gpRANTES; 223 bp, gpIL-8; 512 bp and gpMCP-1; 470 bp. Products were separated by electrophoresis on a 1.5 % agarose gel containing 1 mg/ml ethidium bromide to visualise the DNA.

3.1.2. Preparation and validation of single-stranded DIG-labelled riboprobes

Single stranded riboprobes are the considered to be the probe of choice for *in situ* hybridisation since they avoid the problems of competition between target tissue RNA and the complementary strand as is the case using denatured double-stranded DNA probes. Furthermore RNA-RNA hybrids are far stronger than RNA-DNA hybrids (Vignaud *et al.* 1994). Therefore, considerable time and effort was invested to generate labelled riboprobes.

pBluescript SK- plasmid containing gpRANTES or eotaxin cDNA was digested overnight with selected restriction enzymes to allow maximal linearisation. Enzymes were selected to allow linearisation on either the T7 or T3 polymerase-side of the insert to enable generation of antisense (complementary to) or sense (identical sequence to mRNA) probes respectively as shown in figure 3A. The linearised plasmid was purified by gel electrophoresis of the restriction enzyme mixture, removal of the gel band containing linearised plasmid (figure 3B, bands running at 4.0 kb) and extraction of the DNA using QIAquick columns (3C). Using electrophoresis, non-linearised and linearised forms of the plasmid were separable by virtue of their shape; the migration of the non-linearised plasmid is inhibited by its circular nature and may account for the faint bands running at around 21.2 kb. This purification step was found to be essential and during earlier attempts to synthesise riboprobes transcription using linearised, but unpurifed, DNA failed to yield a DIG-labelled product. In those circumstances it is possible that contaminating non-linearised plasmid, which would also be transcribed in the T7/T3 polymerase system, might have vastly decreased the amounts of free nucleotide as the polymerases circled the whole plasmid.

It was found that following the restriction and gel purification steps only 1/10 of the starting DNA was retained. Thus a minimum of 10 μg of miniprep plasmid DNA was required to generate the 1 μg of purified, linearised DNA necessary per transcription reaction. A number of initial attempts to synthesis riboprobes using 1-5 μg yielded insufficient quantities of the riboprobe for *in situ* analysis.

The quantity of DIG-labelled RNA generated from the transcription of 1 μg of plasmid DNA was quantified by dot blot analysis and compared to a sample of DNA of known concentration. The transcription reaction generated approximately 50 ng/ml DIG-labelled cRNA for both RANTES sense and antisense probes and 25 ng/ml for both eotaxin probes (figure 3D).

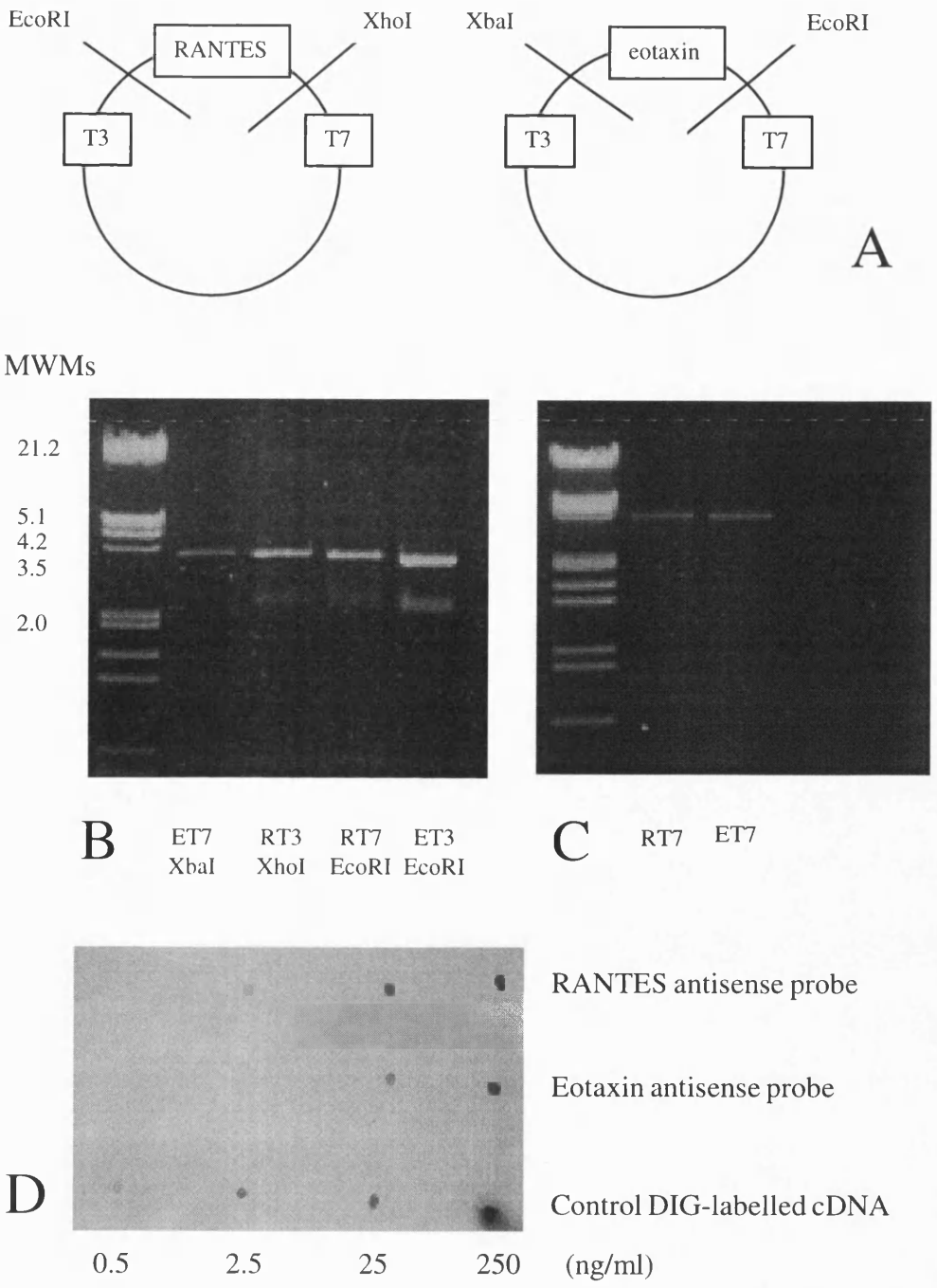


Figure 3. Preparation of DIG-labelled riboprobes. pBSK- plasmid containing RANTES (R) or eotaxin (E) cDNA was linearised with appropriate restriction enzymes either side of the T7 or T3 polymerase sites (A). The digests were separarted by electrophoresis (B) and purified using QIAquick extraction cloumns to obtain pure linearised plasmid (C) which was transcribed using with T7 or T3 RNA polymerases. Incorporation of DIG-11-UTP was confirmed by dotting 1 µl of serial dilutions of the probe onto nitrocellulose and developing using anti-DIG Fab fragments and BCIP/NBT substrate. This enabled quantification by comparison to control labelled DNA of known concentration (D).

3.2. Northern blot analysis of whole lung tissue following OA challenge

3.2.1. Methods of extracting total RNA from guinea pig lung tissue

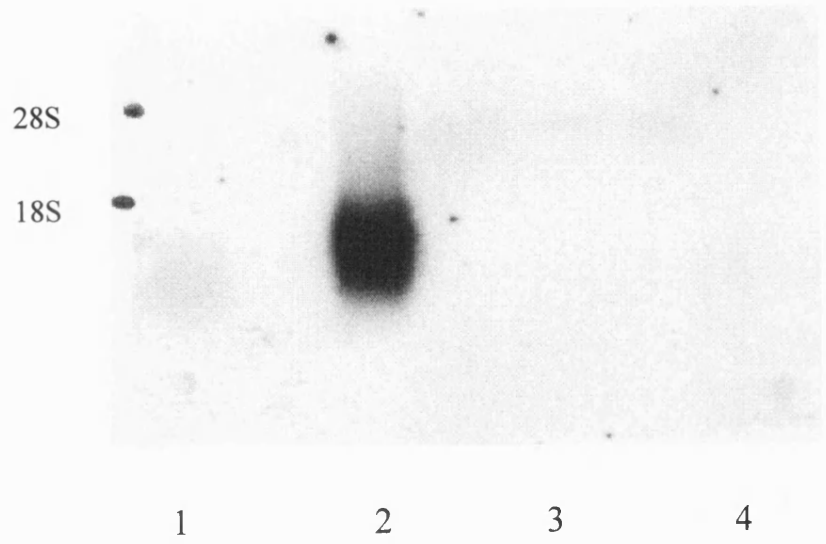
A number of methods were employed to isolate RNA from guinea pig whole lung and substantial time and effort were invested for this purpose. Initial attempts involved the extraction of RNA from frozen pieces of lung tissue using a 4 M guanidine isothiocyanate buffer, followed by a series of phenol/chloroform steps and propan-2-ol precipitation (referred to as method A in 'Materials and Methods'). Visualisation of the RNA on ethidium bromide gels showed a smearing of products which is indicative of RNA degradation. Moreover, there was no evidence of 18 and 28 S bands. Some slight improvement in the purity of the RNA was observed using tissue crushed under liquid nitrogen, since faint 18 and 28 S bands were visible (method B).

During the course of the my studies, a number of commercial reagents became available to aid the extraction of RNA from tissues. RNA extracted from lung tissue crushed under liquid using RNAsol B proved the most successful of all methods attempted, as assessed by the calibre of the 18 and 28 S bands (method C). It is possible that the speed of RNA isolation using such commercial preparations is a major factor since the time the RNA is in a soluble form, when it is most susceptible to RNase activity, is reduced. However, considerable variation in the integrity of RNA was observed between experiments, even using this technique.

3.2.2. Optimal hybridisation conditions for DIG-labelled cDNA probes

The optimal conditions for the hybridisation of RNA to DIG-labelled probes were assessed by probing different blots of equal quantities of the same RNA sample with a probe for gpIL-8 under different buffers and temperatures. Hybridisation to the probe occurred only when using high SDS buffer at 50 °C (figure 4, lane 2). It would appear that temperature is critical since only very slight hybridisation was observed at 42 °C (figure 4, lane 1).

Hybridisation of RNA to the DNA probes using other samples of fibroblasts and lung tissue was also unsuccessful using standard buffer either with or without formamide. In comparison, the use of high SDS buffer at 50 °C produced a distinct signal in both cell line and tissue samples, with no evidence of non-specific binding (see figures 5 and 19).



Lane 1	High SDS buffer at 42°C
Lane 2	High SDS buffer at 50°C
Lane 3	Standard buffer at 42°C
Lane 4	Standard buffer (+50% formamide) at 68°C

Figure 4. Optimisation of hybridisation conditions for DIG-labelled cDNA probes. RNA was extracted from $5x10^6$ guinea pig lung fibroblasts and divided equally between each lane. Following transfer, membrane lanes were incubated with gpIL-8 probe under the described conditions and bound probe was detected using anti-DIG Fab fragments and chemiluminescence substrate.

3.2.3. Results from Northern blot analysis of OA challenged whole lung tissue

The kinetics of chemokine mRNA expression was examined in lung from OA-sensitised and naïve guinea pigs at various time points following aerosol challenge with OA. Low levels of both RANTES and MCP-1 mRNA expression were observed in naïve non-challenged guinea pig lung (figure 5). RANTES mRNA was detected in naïve lung samples following OA challenge at the time points examined. A slight increase was observed in sensitised lung tissue at 2 h post-OA challenge compared to time matched controls but this trend was not consistently seen in all sensitised lungs at later time points. The same lung samples were also examined for expression of MCP-1. In contrast with RANTES, MCP-1 message was greatly elevated in two of three sensitised lungs at 2 h following OA challenge compared to controls, and this increase was also observed at 6 and 12 h post challenge. At 24 h, levels of MCP-1 mRNA were elevated in naïve OA challenged lungs. However accurate assessment of chemokine mRNA levels in sensitised tissue was not possible at this time point due to the under-loading of total RNA as assessed by the ribosomal 18 and 28 S bands (figure 5, lower panels). This may be due to RNA degradation since RNA from equal amounts of starting tissue (25 mg) was loaded in each gel lane. RNA degradation results from exposure to RNases but since intact total RNA was successfully purified from primary and cell line fibroblasts (for example see figures 18 and 19), it seem unlikely that the tissue degradation was due to exogenous RNase contamination. The purification technique may not have adequately remove all endogenous RNases from some tissue samples. In further studies, attempts to assess the comparative time course of expression of RANTES and other chemokine mRNA by Nothern blotting proved inconclusive due to RNA degradation in some samples. For this reason, a semi-quantitative RT-PCR method was developed.

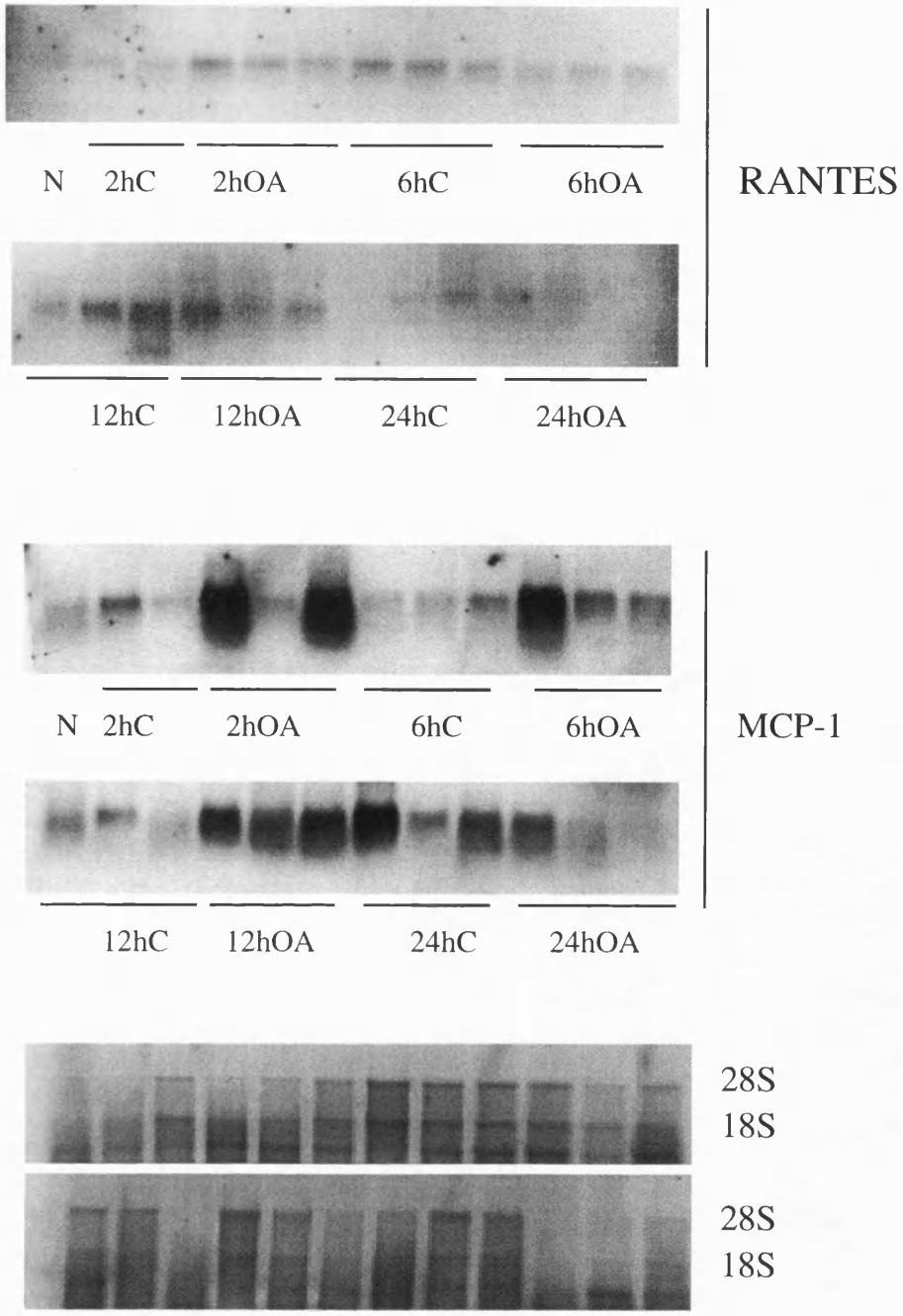


Figure 5. Northern blot analysis of RANTES and MCP-1 mRNA expression in OA sensitised (OA) and naïve (C) guinea pig lung following OA-challenge. At all time points n=3 animals except naïve OA-challenge at 2 h which was n=2. Similar levels of RANTES expression was observed in both sensitised and naïve lungs following OA challenge in comparison to MCP-1 which was upregulated at 2, 6, and 12 h in sensitised samples. 'N' represents mRNA levels in naïve non challenged guinea pig lung. The lower panels show levels of total RNA loading through ribosomal 18 and 28S bands. Similar patterns of expression were obtained in a separate experiment using different sets of n=3 guinea pigs per treatment.

3.3. Semi-quantitative analysis of OA challenged lung by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR).

3.3.1. *Optimisation of PCR using chemokine reverse transcripts from guinea pig lung*

Ovalbumin challenged guinea pig lung tissue was analysed by RT-PCR for the expression of chemokine message. mRNA was extracted from the tissue using Oligo (dT) columns and reverse transcribed into cDNA. In all cases, sufficient amounts of amplified cDNA to detect on a 1 % agarose gel were obtained following 35 PCR cycles and in the prescence of 2 mM $MgCl_2$. The concentration of Mg^{2+} appeared to be important for the amount of DNA produced since decreasing amounts of Mg^{2+} reduce the yield of DNA (figure 6A). Concentrations in excess of 2.5 mM may induce non-specific amplifications ('Introduction to PCR' manual, Perkin Elmer). When total RNA was used as the starting material for the reverse transcription reaction, no amplified cDNA was detected following PCR.

A co-amplification of RANTES cDNA with house-keeping gene β-actin in the same PCR tube, was successfully achieved using the two sets of primers selected (figure 6B). Amplification of reverse transcripts for MCP-1 was only possible in the absence of the selected β-actin primers (figure 6B) and thus the β-actin amplification was run simultaneously in a separate tube. It is possible that the inability of MCP-1 to co-amplify with β-actin was due to primer dimerisation between the pairs selected. Interestingly, despite the use of two separate sets of guinea pig eotaxin primers (see sequences on page 87), no amplification of reverse transcribed cDNA from guinea pig lung was detected either when the eotaxin primers were used alone or in conjunction with β-actin.

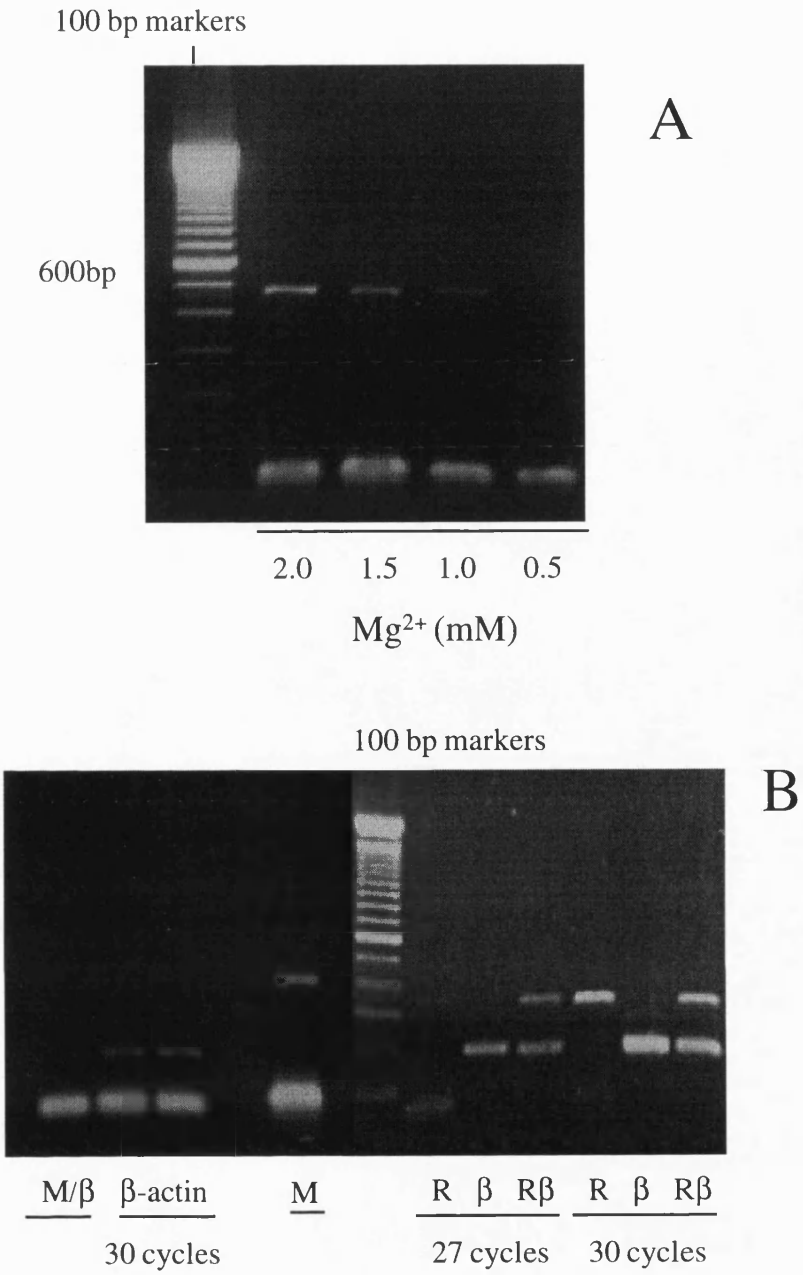


Figure 6. Initial RT-PCR analysis using mRNA from guinea pig whole lung tissue indicated that increasing amounts of Mg^{2+} increased the yield of PCR product using primers for gpMCP-1 over 30 cycles (A). The example shown represents RNA from OA sensitised lung at 24 h following challenge. A similar effect was noted using RNA from a different lung sample from this treatment group. RANTES reverse transcripts (B *right*) could be co-amplified with β-actin and this effect was noted using 27 and 30 cycles. However co-amplification of MCP-1 and β-actin using the selected primers was not successful (B *left*) although either could be amplified alone successfully. The same effects were observed on repeating these experiments. Amplifications with one primer set: R=RANTES, M=MCP-1, β=β-actin. Amplifications with two set: R/β=RANTES and β actin, M/β=MCP-1 and β–actin.

3.3.2. Validation of method

For each lung sample, PCR amplification of 0.3 μl, 1.5 μl and 3 μl of the RT product yielded different amounts of final cDNA and when analysed by electrophoresis and the resulting bands measured by densitometry, this relationship was found to be linear (figure 7). It was observed that increasing amounts of RT product in the PCR reaction eventually caused a plateau in the amount of cDNA produced, highlighting the need to titrate each sample to allow accurate quantification. Product densities were linear within the above range when amplified using primers for either RANTES, MCP-1 or house-keeping gene β-actin which served as a standard. The ratio of PCR product, as measured by densitometry between RANTES or MCP-1 and β-actin at a constant volume of RT product (1.5 μl was selected), allowed standardisation of the target cDNA levels relative to those of the house-keeping gene and thus enabled cross sample comparison using the Mann-Whitney U test.

3.3.3. Results of RT-PCR analysis of OA challenged guinea pig lung

No differences in the levels of RANTES expression relative to β-actin were observed between naïve or OA-sensitised guinea pig lung following OA-challenge at either 2 or 24h. In contrast, the standardised levels of MCP-1 at 2 and 24h were elevated in sensitised OA-challenged lungs compared to time-matched controls (figure 8). Analysis using Mann-Whitney U test revealed that these differences in MCP-1 levels were significantly different ($p<0.01$) at both 2 and 24h.

To ensure that the DNA amplifications did not originate from genomic DNA carried over from the original mRNA extraction, an equivalent volume (ie. 3 μl) of the reverse transcription reaction mixture which had not undergone reverse transcription, was subjected to PCR amplification. Three or four lung samples were selected at random from each of the four treatment groups (naïve and OA sensitised at 2 and 24h post-OA challenge). No samples demonstrated any evidence of amplification suggesting that the results obtained in figure 6 were not due to genomic carry over (figure 9).

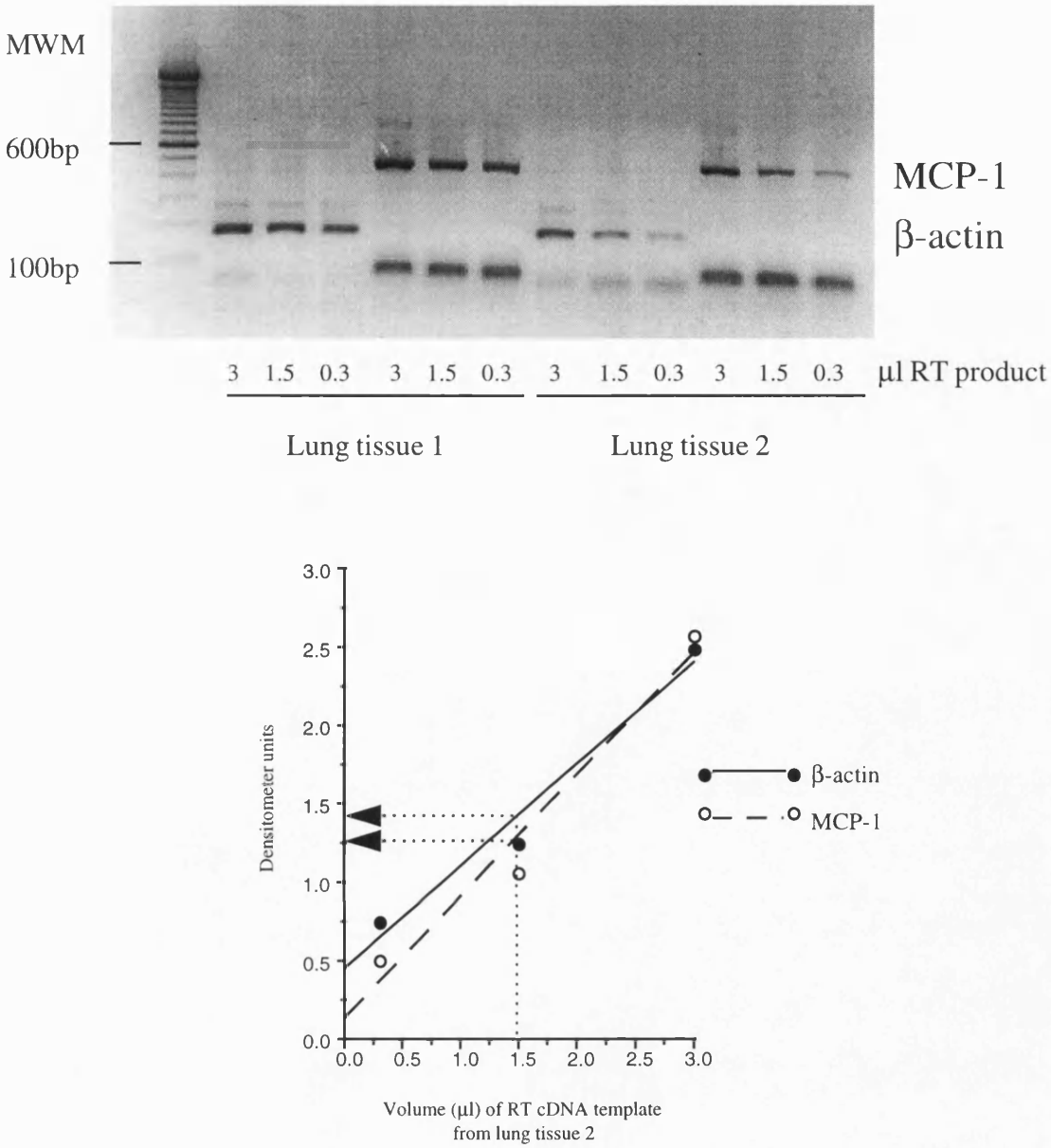


Figure 7. Example of semi-quantitative PCR analysis of MCP-1and β-actin reverse transcripts in guinea pig whole lung samples. Linearity of the amplification was identified by PCR of serial dilutions of the RT cDNA for each lung sample, yielding proportional amounts of product as detected by electrophoresis using a 1 % agarose gel. Examples of MCP-1 amplification from two lung samples are shown (*top*). PCR primers were selected to amplify a 470 base pair fragment of the MCP-1 sequence which could be readily distinguished from the 176 base pair product for β-actin. Amplifications of RANTES cDNA yielded a product of 223 base pairs using the selected primers. The ratio of densitometry measurements of the gel bands between MCP-1 and β-actin at a suitable midpoint along a best fit line (1.5 µl of RT cDNA) enables standardisation of the sample. An example of an analysis using lung sample 2 is shown (*bottom*).

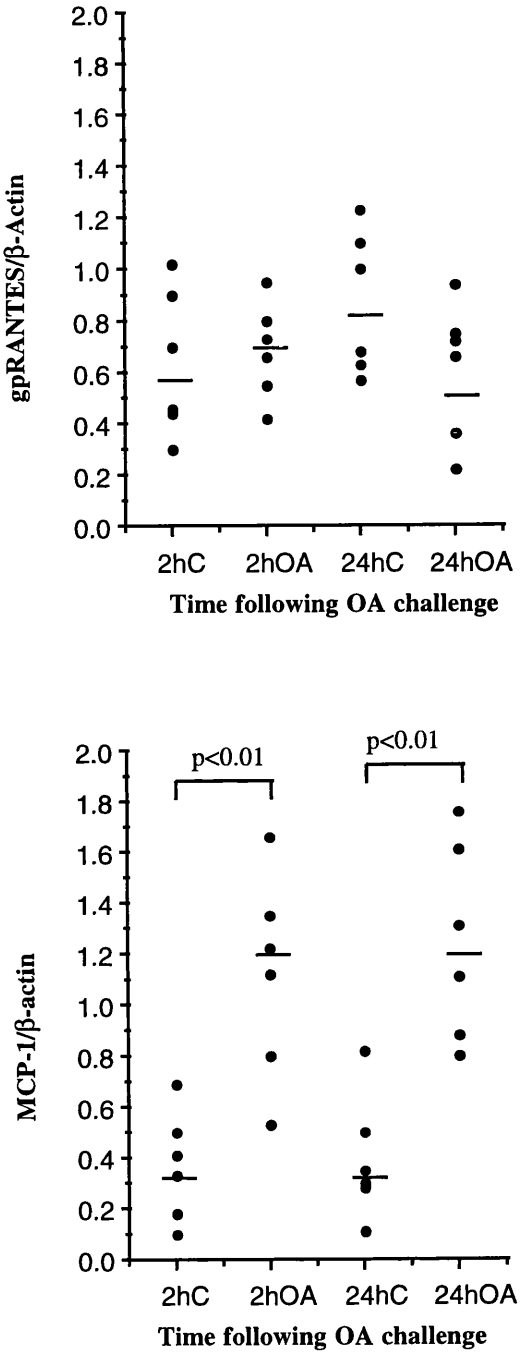


Figure 8. Semi-quantitative RT-PCR analysis of RANTES and MCP-1 mRNA levels in guinea pig naïve (C) and sensitised (OA) lung at 2 and 24 h post OA-challenge. mRNA was extracted from whole guinea pig lung, reverse transcribed and the resulting cDNA amplified using selected primers. Levels of RANTES or MCP-1 amplified product were standardised through β-actin levels in the same sample. Each dot represents an individual animal and n=6 animals were analysed per time point per treatment. Standardised levels between treatments at the same time points were compared using Mann Whitney U test. Bars represent the median levels in each treatment group.

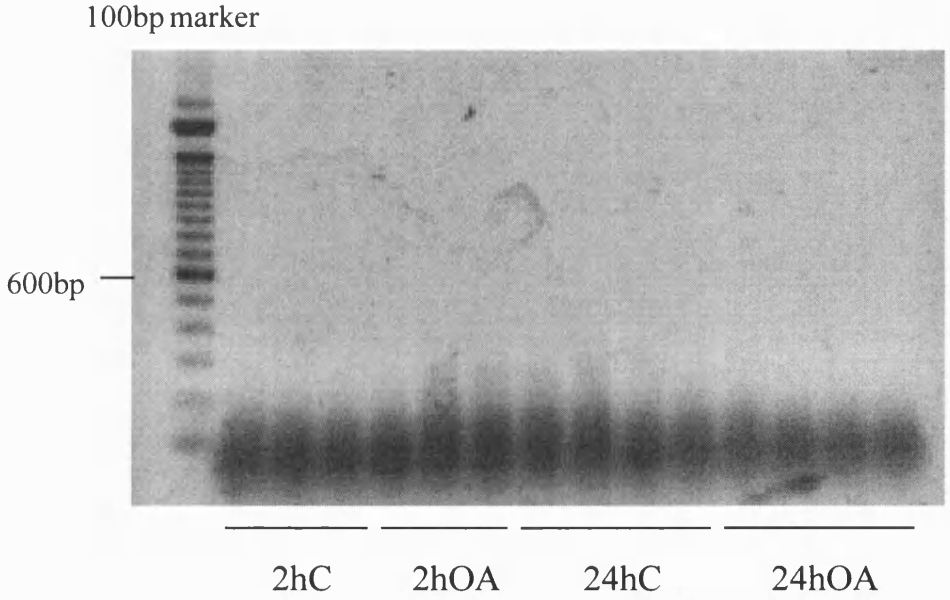


Figure 9. PCR analysis of non-reverse transcribed RT reaction mixure (3 μl) using lung samples from each of the treatment groups selected at random. No evidence of MCP-1 or β-actin amplification was observed using the selected primers, suggesting that there was no detectable carry-over of genomic DNA from the original mRNA preparation. Each lane represents a lung sample from a different guinea pig. This experiment was repeated with the same samples and again no amplifications were observed.

3.4. In situ expression of chemokines in OA-challenged lung

In situ hybridisation was used to assess the effects of OA-challenge on chemokine production from individual cells types in the guinea pig lung. Histological examination revealed that the OA sensitisation and challenge procedure resulted in inflammation in the lung at 24 h, compared to naïve/OA challenged controls, since an eosinophilia was clearly present in the lung parenchyma and within the walls of the bronchi (figure 10).

When these samples were analysed by *in situ* hybridisation using DIG-labelled antisense riboprobes, prominent localisation of RANTES mRNA was observed in the mononuclear cells of naïve OA-challenged lungs which was elevated in two of three sensitised OA challenged tissues studied. Weak staining was also evident in the luminal side of the bronchial epithelium, alveolar epithelium and endothelium (figure 11).

A similar pattern of expression of eotaxin mRNA was observed, with prominent localisation in the mononuclear cells. However, there was no evidence of upregulation in the sensitised guinea pig lung at 24 h post-OA challenged over time-matched controls (figure 12). It is possible that eotaxin mRNA expression in OA sensitised/challenged animals may have been more prominant at earlier time points (3-6h; Jose 1994b) but this was not examined in work for this project. The results were probe-specific since only minimal background staining was found in sense controls for each probe and these were comparable to the endogenous peroxidase activity observed in no-probe control samples.

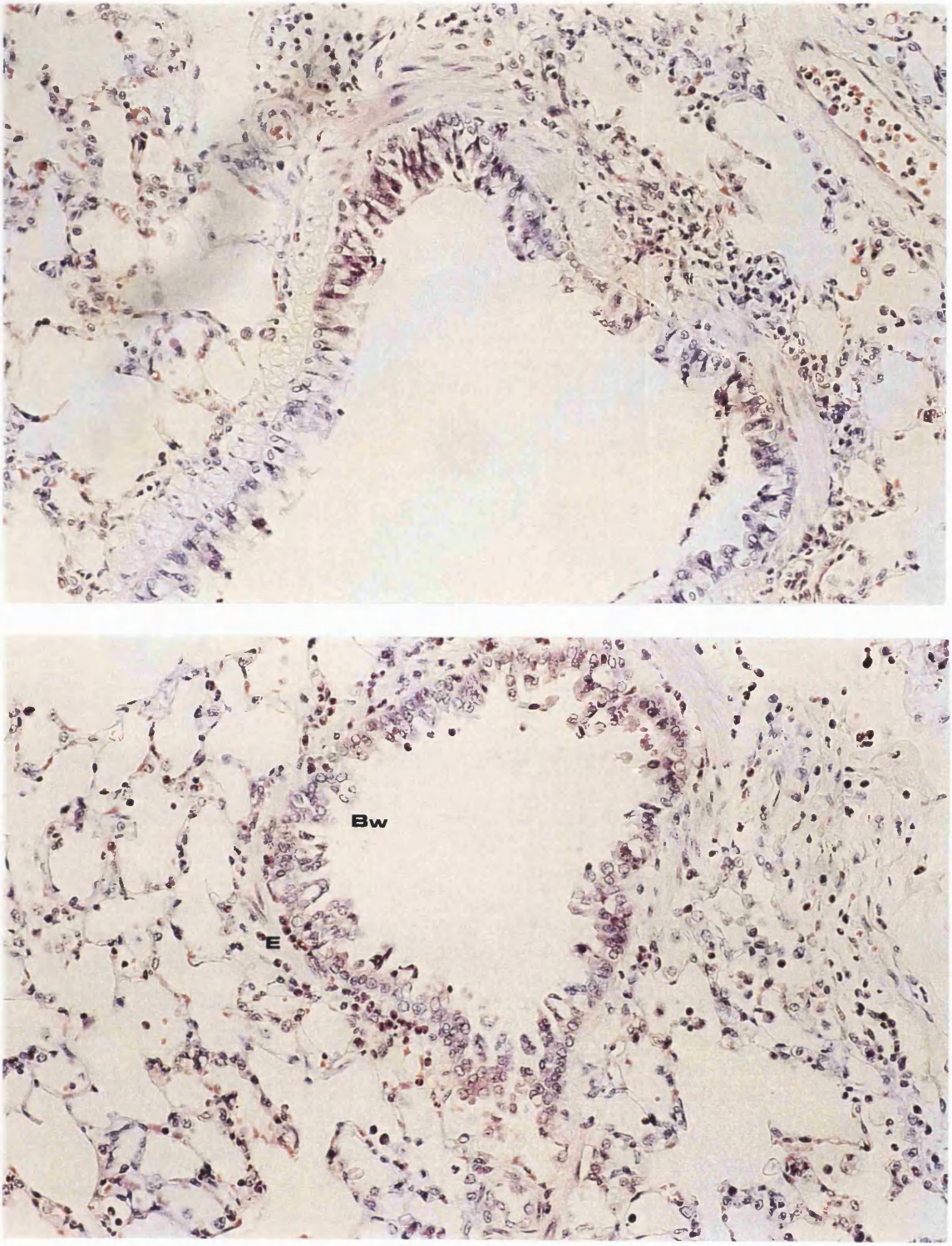


Figure 10. Histological examination of OA-sensitised guinea pig lung tissue revealed a marked monocytic and eosinophilic accumulation (E) especially within the bronchial wall (Bw) in OA sensitised samples at 24 h following OA challenge (*lower photo*) compared to naïve, challenged controls (*upper*). Tissue was stained with haematoxylin and eosin (x200) and sections are shown from representative lungs of OA-sensitised and naive guinea pigs at 24 h following OA challenge; similar effects were also observed in lung sections from two other animals from each treatment group.

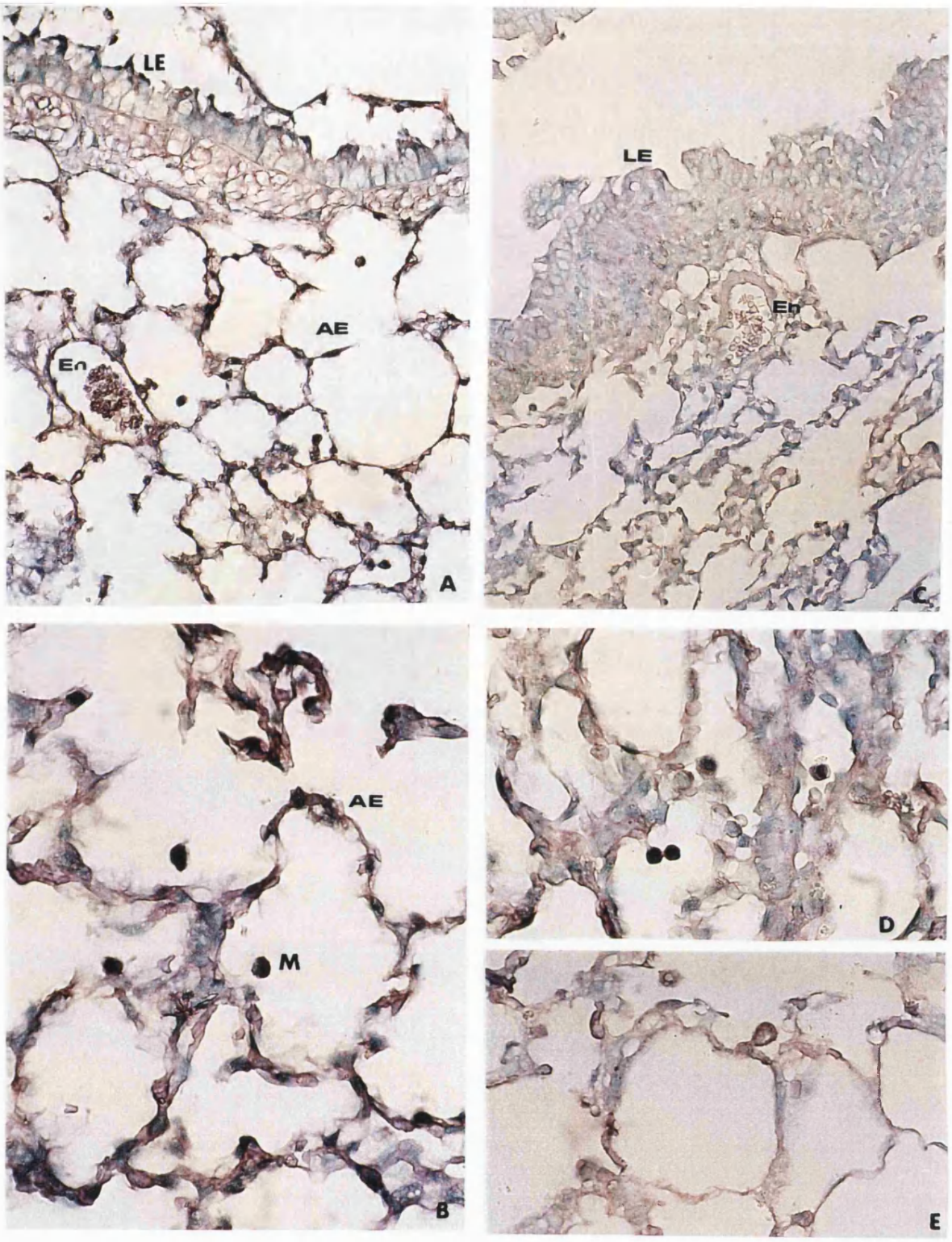


Figure 11. Localisation of RANTES mRNA in the lung of OA-challenged guinea pigs by *in situ* hybridisation using a DIG-labelled antisense riboprobe. RANTES was strongly expressed in macrophages (M) and to a lesser extent in the bronchial lumen epithelium (LE), alveolar epithelium (AE) and endothelium (En) at 24 h in OA sensitised/challenged lung (A x400, B x1000) but less so in naïve/OA challenged animals (C x400, Dx1000). Only minimal background staining was evident using RANTES sense probe (E x1000). Analysis of two other lungs from different naïve/challenged animals indicated similar levels of RANTES as shown above. Analysis of the OA sensitised/challenged animals indicated that RANTES expression was also elevated in a second lung sample from a different animal but not in a third. The hybridisation signal for RANTES is expressed as dark brown and section were counter-stained with methyl green.

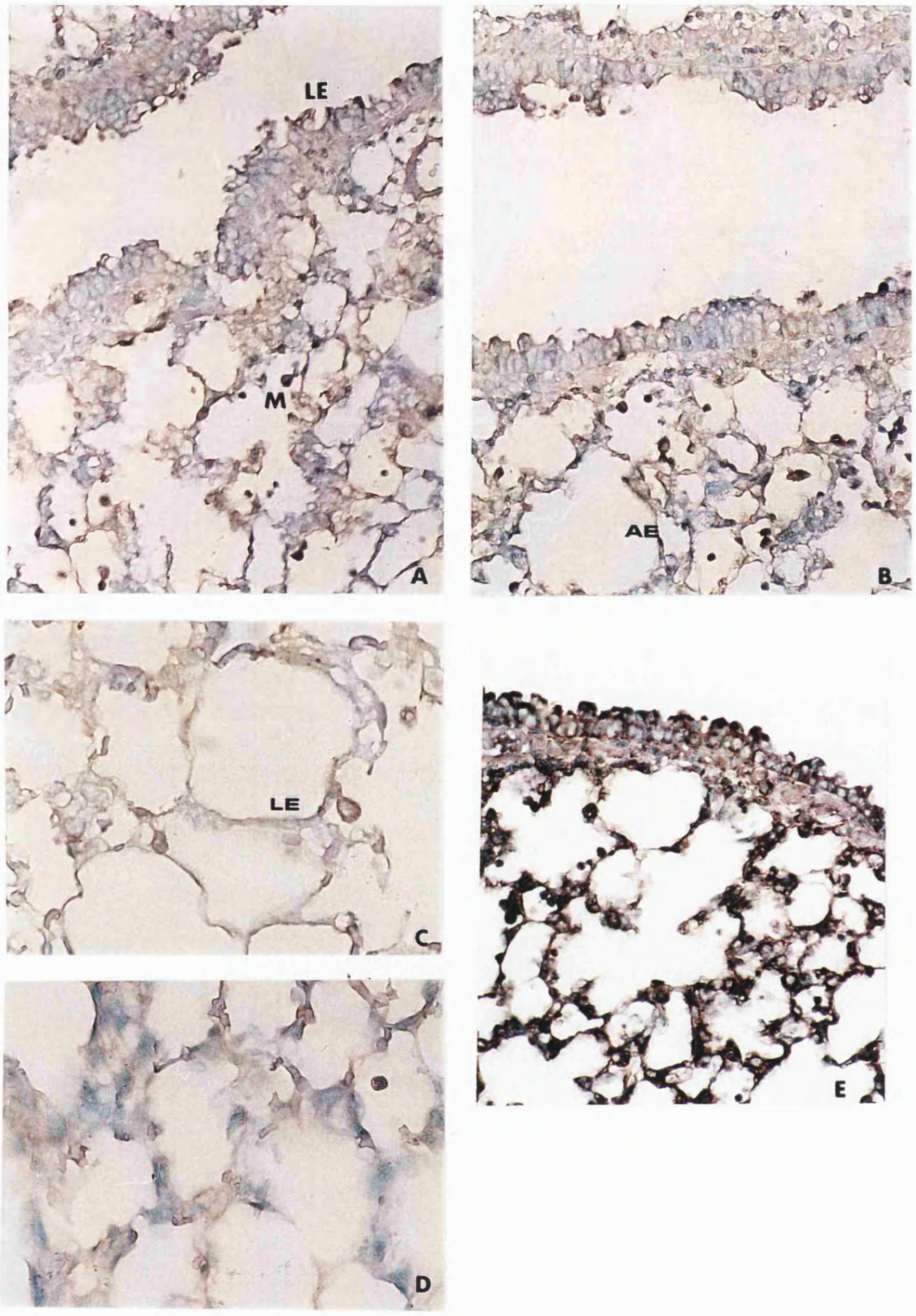


Figure 12. Localisation of eotaxin mRNA in OA-challenged guinea pig lung using *in situ* hybridisation. Eotaxin message was expressed in mononuclear cells (M), bronchial lumen epithelium (LE) and alveolar epithelium (AE) at 24 h in OA sensitised/challenged lung (A x400) but at no stronger levels than in naïve/challenged lungs (B x400). Only minimal background staining was evident for eotaxin sense (C x1000) and no-probe (D x1000) negative controls. Figure E shows localisation of mRNA for β-actin (x400). All section are representative of n=3 animals. Hybridisation signal is expressed as dark brown and sections are counter-stained with methyl green.

3.5. Detection of RANTES protein in the whole guinea pig lung

In an attempt to examine RANTES protein levels in guinea pig whole lung tissue following OA challenge and to avoid the difficulties associated with the handling of RNA, extensive efforts were made to analyse tissue homogenates by Western blotting. A panel of monoclonals raised to human RANTES and polyclonal antisera raised to guinea pig RANTES were used, but in both cases required initial characterisation for their ability to recognise gpRANTES protein.

3.5.1. Characterisation of monoclonal anti-human RANTES mAbs.

The panel of eleven monoclonals were initially screened for their ability to recognise guinea pig RANTES using an ELISA, by absorbing guinea pig RANTES (0.1-100 ng/ml) onto ELISA plates and detecting using different concentrations of the monoclonals (0.1, 1 and 5 μg/ml). At an antibody concentration of 1 μg/ml, mAbs A, E and F recognised gpRANTES by ELISA but only at the highest concentration of gpRANTES examined, 100 ng/ml (figure 13). Absorbance values were no greater using a higher concentration of antibody (5 μg/ml), whilst 0.1 μg/ml was insufficient to detect the protein.

Dot blot analysis (figure 14) confirmed the ability of monoclonal antibodies A and F at 1 μg/ml to recognise gpRANTES, and antibody B did not detect gpRANTES either by ELISA or dot blot analysis. However, antibody E did not detect gpRANTES by dot blot analysis despite a 4.5 fold increase over basal absorbance levels using ELISA.

The panel of antibodies was also used to detect human and gpRANTES run on a 15 % polyacrylamide gel under denaturing conditions (ie. in the presence of 2-

mercaptoethanol). Antibodies A and F recognised both human and guinea pig RANTES but detected 0.1μg of hRANTES with greater efficiency than 1 μg gpRANTES, as assessed by the intensity of the Western detection band. All other monoclonals detected hRANTES but not gpRANTES except J, which recognised neither (figure 15).

A summary of the results for the characterisation of the anti-human RANTES monoclonals is given in table 3.1.

Anti-hRANTES monoclonal	ELISA	Dot blot analysis	Western blot detection of proteins
A	++	+++	hRANTES>gpRANTES
B	–	+	hRANTES only
E	+++	+	hRANTES only
F	+	++	hRANTES>gpRANTES
D, G, H, I, J, L, K	–	Not tested	hRANTES only except for J

Table 3.1. Summary of characterisation of anti-human RANTES monoclonal antibodies for their ability to recognise recombinant gpRANTES

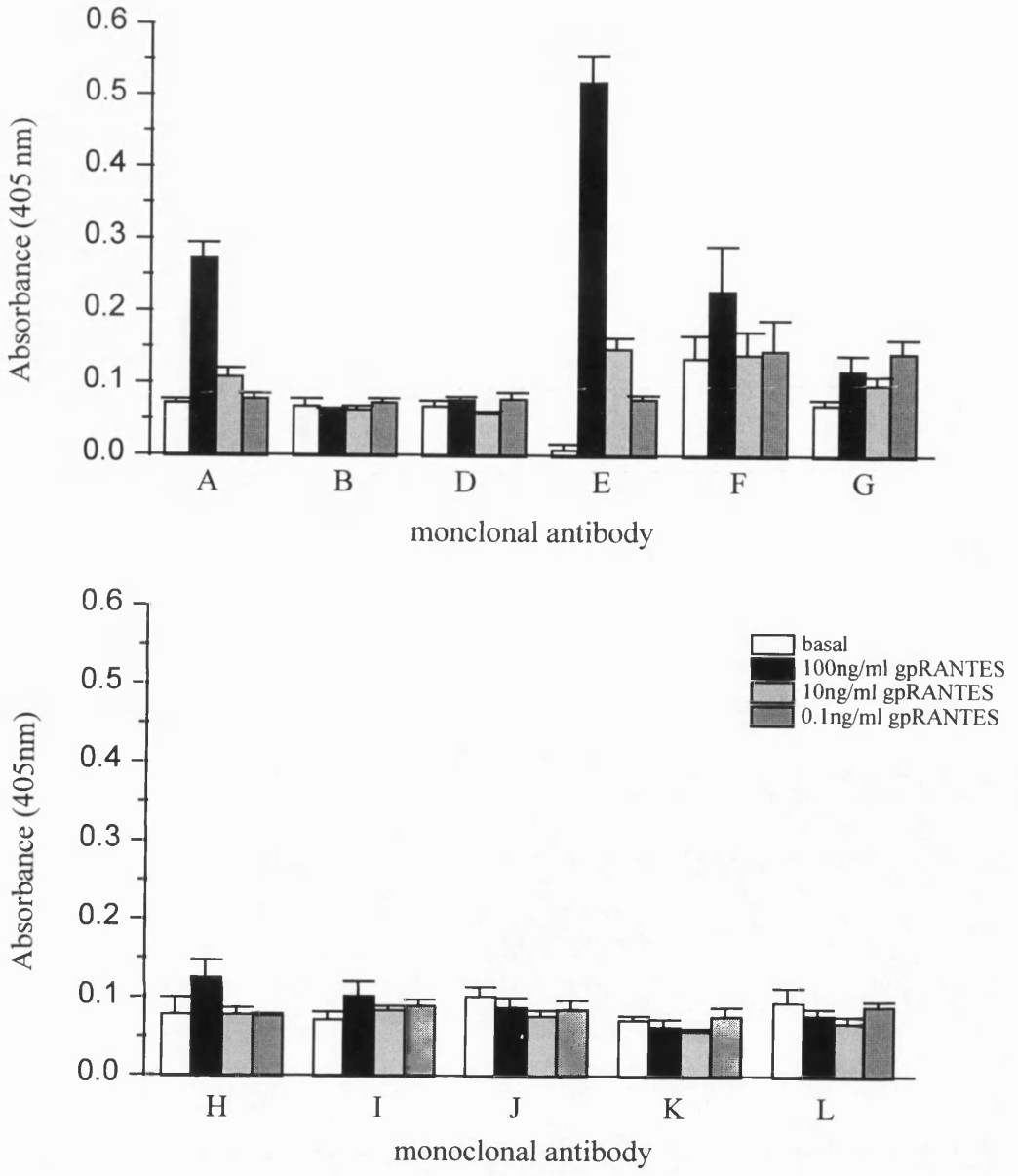


Figure 13. Detection by ELISA of gpRANTES using a panel of anti-human monoclonal antibodies. The ELISA was performed by absorbance of gpRANTES onto 96 well plates followed by incubation with 1 μg/ml of the monoclonal antibody and detecting with rabbit anti-mouse secondary antibody and substrate *p*-nitrophenylphosphate. Each bar represents mean ± SEM for n=3 wells.

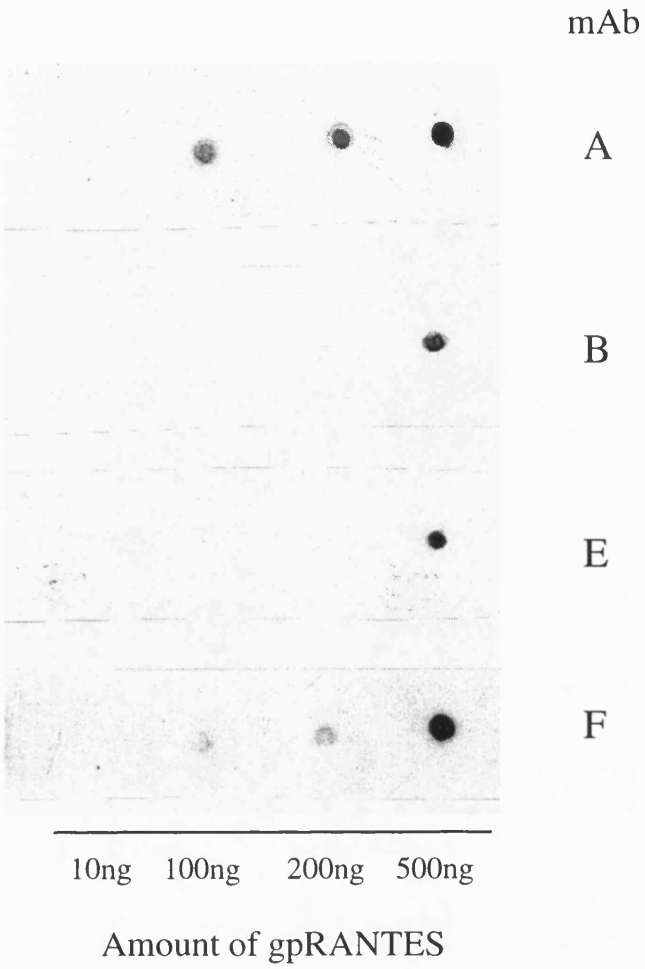


Figure 14. Detection by dot blot analysis of gpRANTES using four of the anti-human RANTES monoclonals. Serial dilutions of the chemokine were dotted as 1 μl, containing the indicated amounts, onto nitrocellulose membrane and incubated with 1μg/ml of the respective monoclonal antibody. Bound antibody was detected using 1/10,000 dilution of goat anti-mouse and ECL reagent.

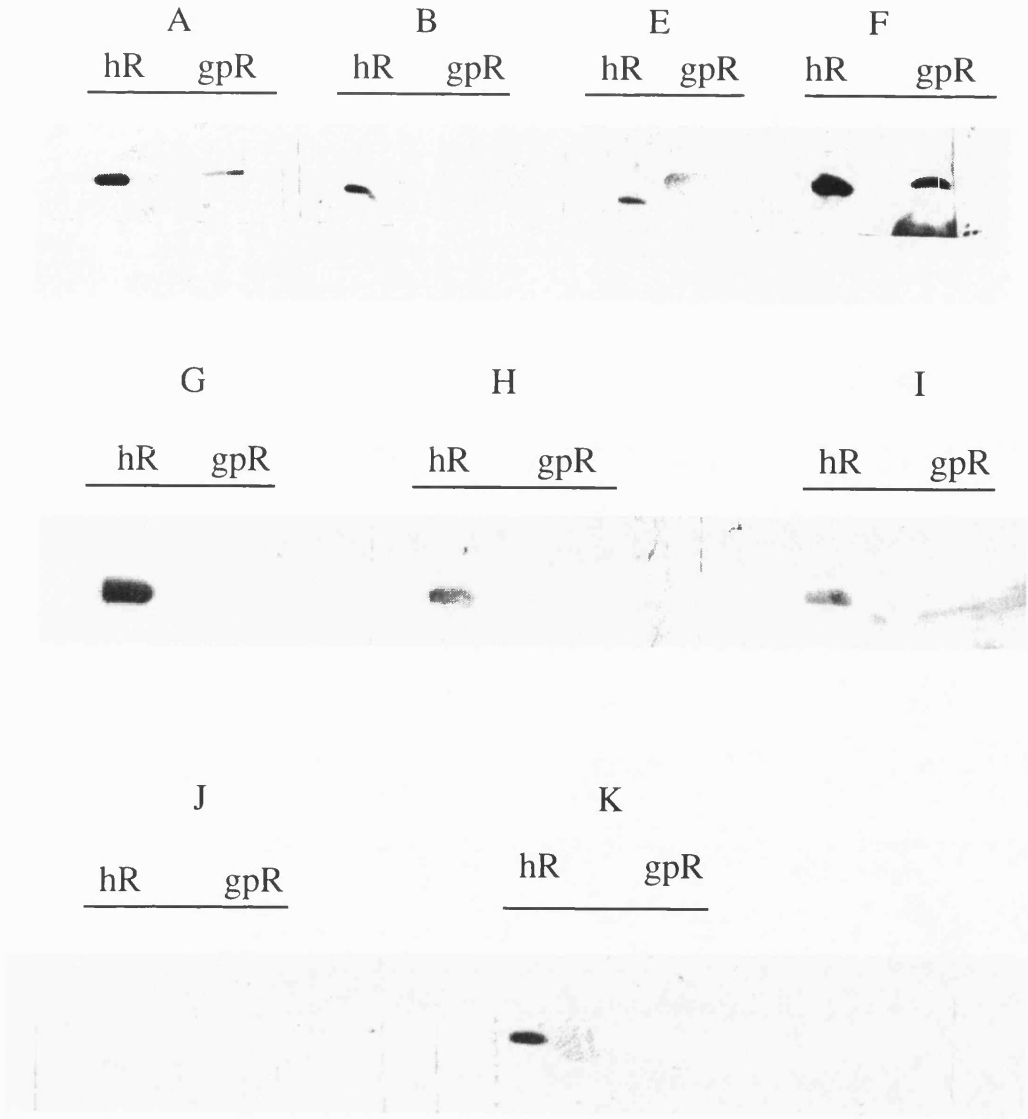


Figure 15. Detection of gpRANTES and hRANTES by Western blot analysis using anti-human RANTES monoclonals. 0.1 μg rhRANTES and 1 μg gpRANTES were run on a 15 % polyacrylamide gel under denaturing conditions. The proteins were transferred and membranes incubated with 1 μg/ml of the respective monoclonal antibody. Bound antibody was detected using goat anti-mouse secondary antibody and ECL.

3.5.2. Western blot analysis of OA challenged lung tissue using selected anti-human RANTES monoclonal antibodies

Two of the anti-hRANTES monoclonal antibodies (A and F) were selected from the above characterisation studies in order to analyse lung tissue homogenates. Protein samples were prepared from OA-sensitised and naïve guinea pig lungs at 2 and 24 h following OA challenge and separated by SDS-PAGE on a 15 % gel. The protein was transferred onto nitrocellulose and membranes were incubated with either antibody A or F (1 μg/ml) and bound antibody detected with goat anti-mouse IgG secondary antibody and ECL. Both antibodies apparently recognised protein of approximately 40-64 kDa (figure 15i. A) but further investigation, probing these same lung samples with secondary antibody only, revealed that these bands were detected because of non-specific binding of the secondary antibody (figure 15i. B). The anti hRANTES mAbs also recognised protein running at approximately 10 kDa which was not due to non-specific secondary antibody binding (15i. A and B). However, this protein ran at a higher molecular weight than recombinant gpRANTES. Furthermore analysis of total protein in these same samples using a Coomassie stained gel revealed that the protein was present in very large quantities in the guinea pig lung (figure 15i. C). This evidence suggested that the protein was probably not RANTES and N terminal sequencing of this band identified this protein to be a component of haemoglobin (E. Magnenat, Glaxo IMB, Geneva).

Despite the fact that both antibodies were able to detect recombinant guinea pig and human RANTES, no RANTES protein was detected in OA sensitised or naïve lung samples either at 2 or 24 h following OA challenge (figure 15i. A). A number of possible reasons may explain this finding. RANTES protein may not be present in these samples, although the same samples did contain RANTES mRNA (see figure 5).

Alternatively, RANTES protein may not be present in sufficient quantities in the extracts for detection and thus requiring purification of the samples, such as size exclusion gel filtration or heparin affinity binding. Furthermore, Western blot analysis demonstrated a marked difference in sensitivity of the antibodies between gpRANTES and hRANTES even though the proteins share 90 % homology (see chapter 5). The ability to raise monoclonals in the mouse against human RANTES that also cross react with gpRANTES probably depends on the monoclonal recognising an epitope which is the same in the human and guinea pig RANTES protein but different in the murine. Alignment of the amino acid sequences (refer to figure 20) shows that this occurs only at residue 3, where tyrosine in the guinea pig and human is replaced by threonine in the mouse, residue 15 (isoleucine is replaced by leucine), residue 17 (arginine is replaced by leucine), residue 24 (isoleucine is replaced by valine), residue 45 (lysine is replaced by arginine), and residue 59 (arginine is replaced by glutamine in the mouse). Further, other sites (residues 16,18 and 32) that differ between murine and human are conserved between murine and guinea pig and it may be that the antibodies raised were against these residues.

3.5.3. Characterisation of gpRANTES antiserum

In an attempt to over come these problems, antiserum raised in rabbits in response to gpRANTES was characterised for its ability to recognise gpRANTES. Serial dilutions of recombinant gpRANTES were dotted onto nitrocellulose as 1 µl and 'strips' of these dilutions were incubated with different dilutions of the antiserum. Bound antibody was detected using goat anti-rabbit secondary antibody and ECL reagent. The antiserum recognised gpRANTES, but not at amounts less than 100 ng (figure 16), which was comparable to that for the monoclonal antibodies (compare with figure 14). Furthermore, since there was no evidence of a dilution effect using decreasing amounts of antiserum, it is possible that the detection of gpRANTES was due to non-specific binding of antibody. For these reasons, analysis using the polyclonal antiserum was not continued.

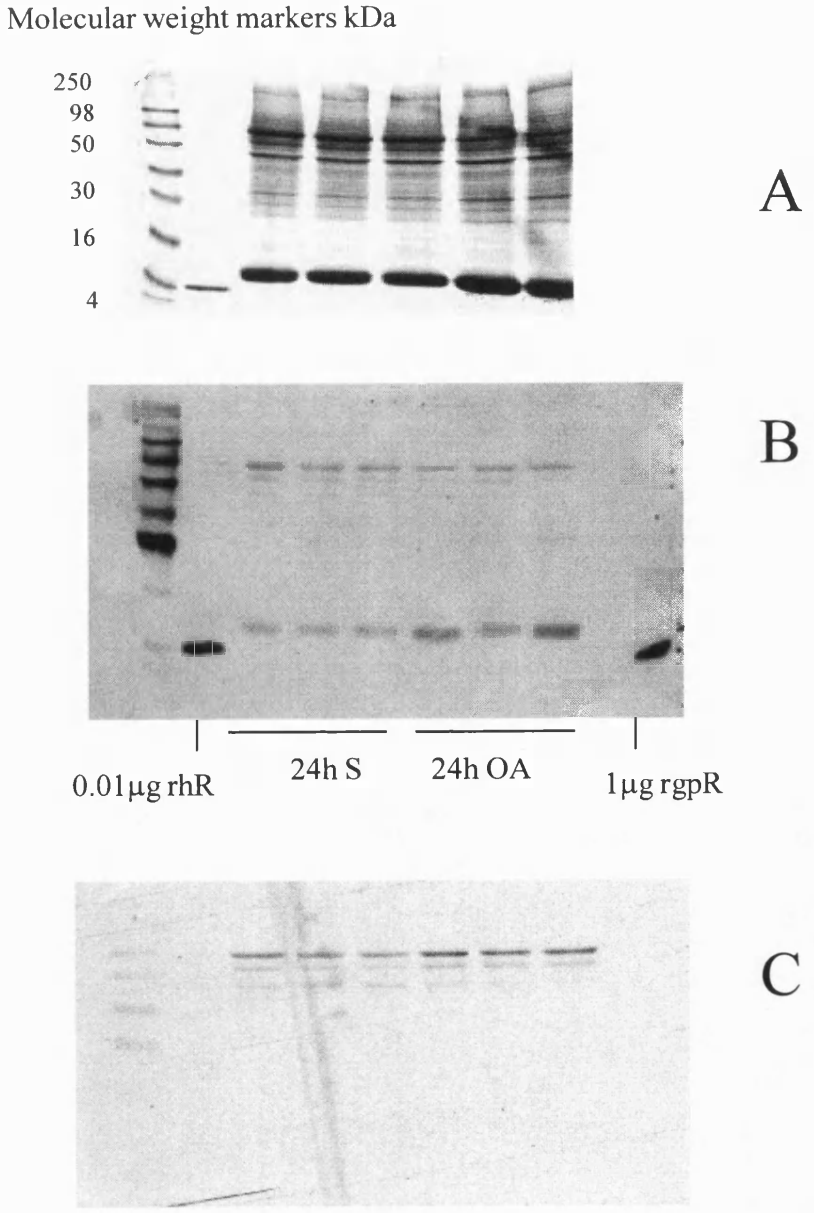


Figure 15i. Western blot analysis of OA sensitised and naïve guinea pig whole lung at 24 h following OA challenge using anti-hRANTES mAb 'A' at 1 µg/ml. Lung homogenates were separated on a 15 % polyacrylamide gel (A). Protein bands detected between 40-64 kDa (B) were due to non-specific binding of secondary antibody (C). Protein of approximately 10 kDa was detected by mAb 'A' but not by secondary antibody alone. This protein was also visible on a Coomassie gel showing total protein (A). Sequencing identified this band to be a component of haemoglobin. No evidence of RANTES protein was detected in any lung samples. Each lane represents lung homogenate from one guinea pig. Similar results were obtained using tissue at 2 h post OA challenge and using the antibody 'F' at 1 µg/ml.

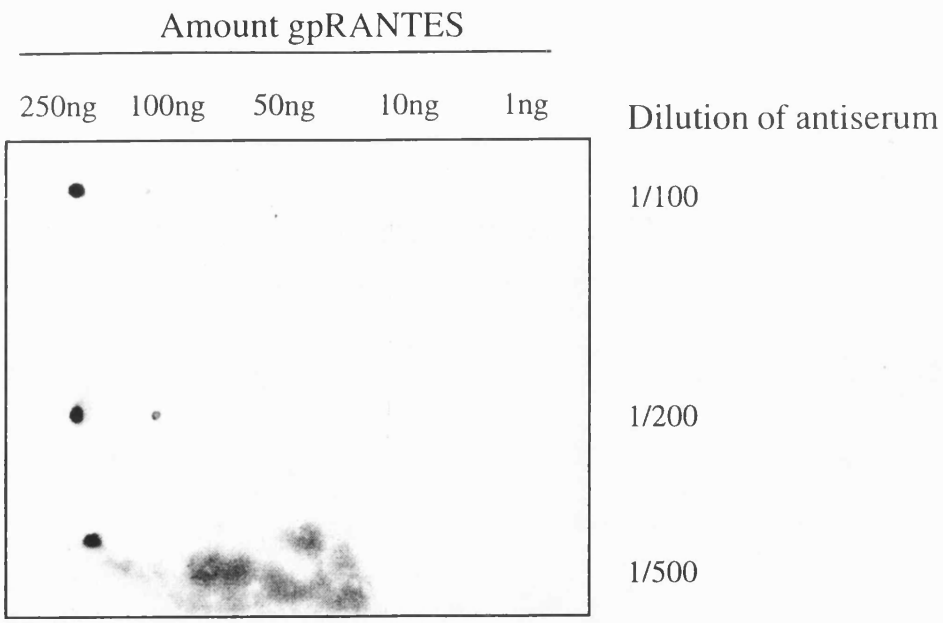


Figure 16. Dot blot analysis of gpRANTES detected using antiserum raised against gpRANTES in the rabbit. Bound antibody was detected using goat anti-rabbit secondary antibodies and ECL.

3.6. Detection of chemokines in isolated guinea pig cells

3.6.1. Expression of RANTES in guinea pig peritoneal macrophages

Isolated guinea pig peritoneal macrophages were stimulated in culture with either LPS, recombinant gpRANTES or vehicle and extracted after 24 h. Preliminary northern blot analysis (this experiment was performed only once) revealed no expression in vehicle control cells but RANTES mRNA was observed following LPS stimulation at all time points tested and at 4 h and 12 h following stimulation with 50 nM recombinant gpRANTES (figure 17).

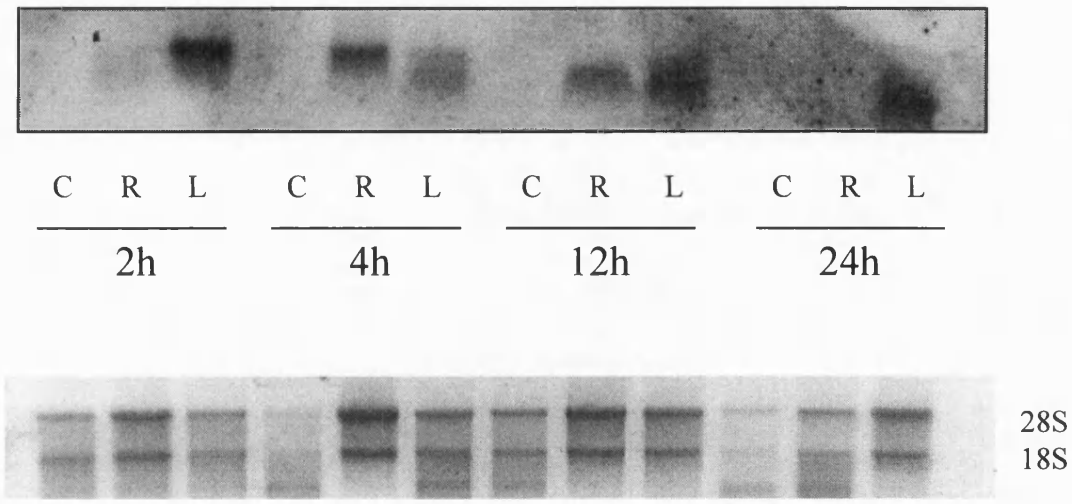


Figure 17. Northern blot analysis for RANTES mRNA from isolated peritoneal macrophages at various times following stimulation. Approximately 10 µg of total RNA extracted from $4x10^6$ cells was loaded per lane. Cells were stimulated at $2x10^6$/ml with 50 nM gpRANTES (R), 1 µg/ml LPS (L) or PBS-vehicle (C) as shown.

3.6.2. Kinetic expression of chemokine mRNA in a guinea pig lung fibroblast cell line and in primary cell cultures.

In an attempt to elucidate other cell types contributing to chemokine production, expression of mRNA was investigated using Northern blot analysis in a guinea pig lung fibroblast cell-line JH4-Cl1 and in cultured cells grown from collagenase digests of guinea pig lung tissue. Outgrowth of adherent cells from the digests was observed at days 2-4 after start of culture and the colonised cells rapidly grew to confluence within a week. Since these cells could only be successfully passaged using trypsin/EDTA solution up to passage 10, experiments were performed on cells between passage 2-6. In the absence of staining techniques these primary cells can not be catagorically identified. However, these cells displayed a spindle shaped morphology typical of cultured fibroblasts and akin to the guinea pig fibroblast cell line, JH4 Cl-1. Furthermore, their morphology was not characteristic of the organised 'swirling' nature of smooth muscle cell cultures or the pavemental appearance of epithelial cells (see below). In addition, analysis of all cultures by light microscopy failed to identify any regions of contaminating cell growth.

Attempts to establish primary cultures of guinea pig airway tracheal epithelium were successful by incubating lung digests with Bronchial Epithelial Growth Medium (Clonetics), but since the cells could not be successfully passaged, sufficient numbers could not be grown. These cells were also adhesive but portrayed a characteristic pavemental appearance in contrast to the cultured fibroblasts.

Confluent cultures of the JH4-Cl1 cells did not constitutively express mRNA for IL-8, MCP-1 or RANTES and no induction was observed following stimulation with human

recombinant TNFα (30 ng/ml) and/or rhIL-1 (10 ng/ml). However, incubation with medium from LPS-stimulated guinea pig peritoneal macrophages, which was assumed to be a rich source of guinea pig cytokines ('cytokine supernatant'), stimulated a time-dependent expression of MCP-1 in the JH4-Cl1 cells. Evidence of a biphasic expression was observed peaking at 2 and 24 hours compared to the negligible expression in time matched controls. A similar pattern of expression was observed for IL-8 (figure 18).

MCP-1 expression was observed in primary lung fibroblasts at 4 and 12 h which was not increased following stimulation with the cytokine supernatant from LPS-stimulated macrophages and Con A-stimulated spleen cells. However, at later time points (24 and 48 h) expression of MCP-1 was stimulus-dependent (figure 19). This difference could reflect a time dependent response to FCS used in the medium which indueced MCP-1 expression at early but not later time points. As for the JH4-Cl1 cell line, only weak expression of MCP-1 was observed at time points post 24 h stimulation although this effect did not seem to be related to cytotoxicity as assessed by trypan blue exclusion. Examination of the same primary fibroblasts for the presence of RANTES and MIP-1α mRNA revealed no expression at any time point analysed even following stimulation with the cytokine supernatant. To assess the expression of eotaxin, a DIG-labelled oligoprobe (R&D systems, Abingdon, UK) was used. The eotaxin probes did detect some distinct bands but on closer inspection these were found to align with ribosomal 28 and 18 S RNA and therefore are probably due to non-specific binding (figure 19). This experiment was attempted a further two times. In each case, expression of MCP-1 but not RANTES, eotaxin or MIP-1α was observed.

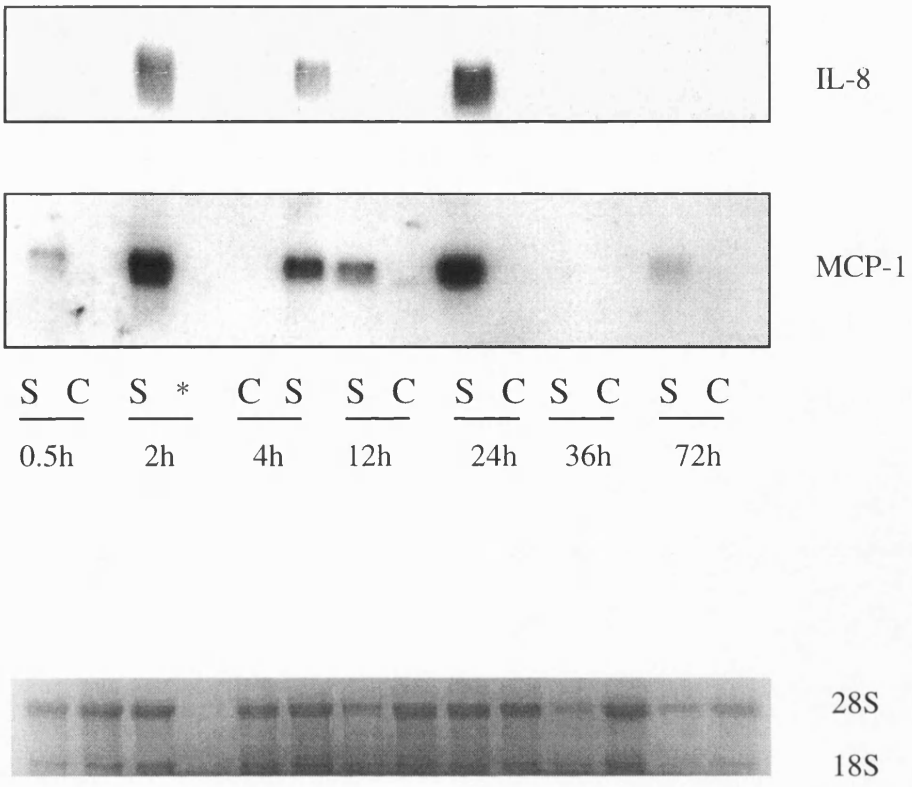


Figure 18. Time course of expression of IL-8 and MCP-1 mRNA in a guinea pig lung fibroblast cell line, JH4-Cl1 stimulated with cytokine supernatant (S) or vehicle (C). Ribosomal 18 and 28S bands show loading of total RNA. Chemokine mRNA was detected using full length DIG-labelled cDNA probes to gpIL-8 or MCP-1. * 2 h C not tested.

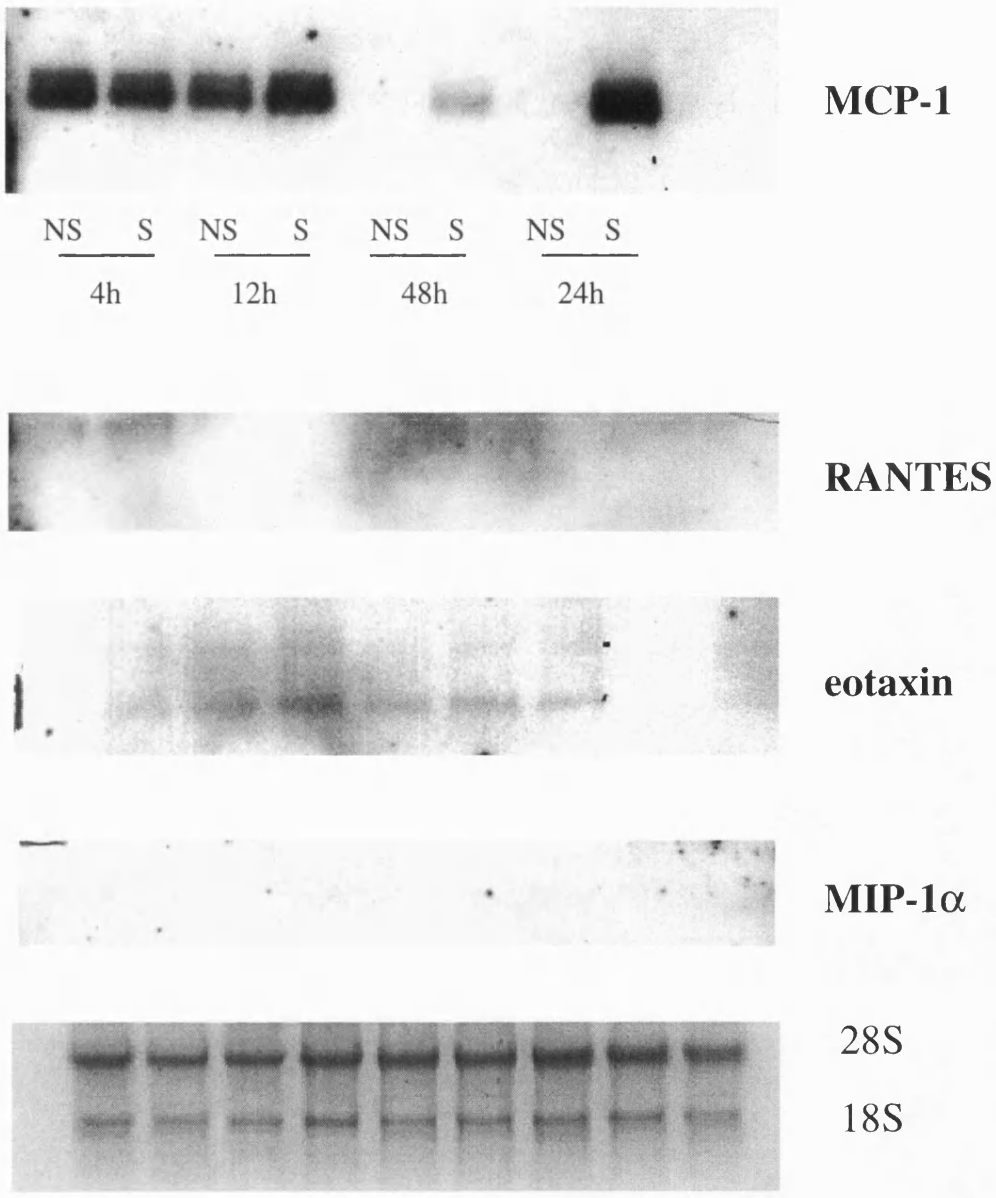


Figure 19. Chemokine expression in primary lung fibroblast-like cells following stimulation and detected by DIG-labelled full length cDNA probes, except for eotaxin, where a DIG-labelled oligo probe was used. MCP-1 mRNA expression was observed at 4 and 12 h (lanes 1-4) and also at 24 h (7 & 8) and 48 h (5 & 6) but only following stimulation with cytokine supernatant (S). 'NS' represents mRNA levels in the absence of cytokine supernatant. In comparison, RANTES, eotaxin (bands correlate with 18/28S ribosomal RNA) or MIP-1α mRNA expression was not detected in these cells.

3.7. Summary of results: chemokine detection in the guinea pig lung

- Both RANTES and MCP-1 mRNA were expressed in the naïve guinea pig lung
- In a guinea pig model of allergic lung inflammation, RANTES and MCP-1 mRNA were differentially expressed. RANTES mRNA was not increased in OA sensitised/challenged lungs compared to levels in naïve/challenged lungs. In comparison, MCP-1 mRNA was upregulated in OA sensitised samples following challenge.
- Attempts to use murine and human RANTES monoclonal antibodies or rabbit anti-guinea pig RANTES antiserum to analyse guinea pig RANTES protein in lung lysates were unsuccessful.
- *In situ* hybridisation identified mononuclear cells and to a lesser extent bronchial epithelium and endothelial cells as potential sources of both RANTES and eotaxin.
- However, no expression of RANTES, eotaxin or MIP-1α mRNA was detected in primary fibroblast-like cells. These cells and the guinea pig lung fibroblast cell line, JH4-Cl1, did express MCP-1 and IL-8 mRNA in response to cytokine supernatant derived from LPS-stimulated guinea pig macrophages.

CHAPTER 4: RESULTS

The expression and purification of recombinant guinea pig RANTES protein

4.1. The cloning of gpRANTES cDNA

The gpRANTES cDNA was isolated from a library constructed from concanavalin A-stimulated guinea pig spleen cells. As shown in figure 20, the gpRANTES cDNA comprises 516 base pairs with an open reading frame encoding a 92 amino acid protein. This includes a presumably cleavable leader sequence akin to that described for hRANTES (Schall *et al.* 1988) and it was assumed that the point of cleavage in the guinea pig protein is the same. This gives rise to a 68 amino acid protein with a predicted molecular weight of 7901 Da for gpRANTES. gpRANTES shows 90% nucleotide sequence identity with hRANTES (Schall *et al.* 1988). The deduced amino acid sequence has 87% and 86% homology to human and murine RANTES (Schall *et al.* 1988; Schall *et al.* 1992) respectively (figure 20) with 91% identity to hRANTES in the region encoding the presumed 68 amino acid mature protein.

```
  1  GCCCCTGCCT GCAAGTACCA TGAAGGTCTC CGCAGCTGCC CTCTGTGTCA

 51  TCCTCACCAC TGCTGCCCTC TGTGTTCCTG CATCTGCCTC CCCATATGCC

101  TCAGATACCA CTCCTTGCTG CTTTGCCTAC ATCTCCCGGG CACTGCCCCG

151  CACCCACATC AAGGAATATT TCTACACCAG CAGCAAGTGC TCCAACTTAG

201  CAGTCGTATT TGTTACCCGA AAGAACCGCC AGGTGTGTGC CAACCCAGAG

251  AAAAAGTGGG TTCGAGAGTA CATCAACTCT TTGGAGATGA GCTAGGAGAA

301  GAACCACCTT GAACCTGACC TTGTAGAAAC TCCGCTGCTG CTGCTTGTTC

351  TGGTCTTAAC CAGTTTAGGA AACTTCTCAA TCCCTACTCT CATTCATCCT

401  TGGGAGGGCA TGGATTCTAT TACAACACAG CAATGCTAGC AGCTTCTCCC

451  ACTTAGAGGC AAAAGAGCTA TCGAAGCCCT GCCGTGGCCA TGGGAAGTCT

501  CTAGGTTCCC AGGCC
```

	-23		
guinea pig	**MKV**	**SAAALCVILT**	**TAALCVPASA**
human	MKV	SAARLAVILI	ATALCAPASA
murine	MKV	SAAALTIILT	AAALCTPAPA

	1	11	21
guinea pig	**SPYASDTTPC**	**CFAYISRALP**	**RTHIKEYFYT**
human	SPYSSDTTPC	CFAYIARPLP	RAHIKEYFYT
murine	SPTGSDTTPC	CFAYLSLALP	RAHVKEYFYT

	31	41	51
guinea pig	**SSKCSNLAVV**	**FVTRKNRQVC**	**ANPEKKWVRE**
human	SGKCSNPAVV	FVTRKNRQVC	ANPEKKWVRE
murine	SSKCSNLAVV	FVTRRNRQVC	ANPEKKWVQE

	61
guinea pig	**YINSLEMS**
human	YINSLEMS
murine	YINSLEMS

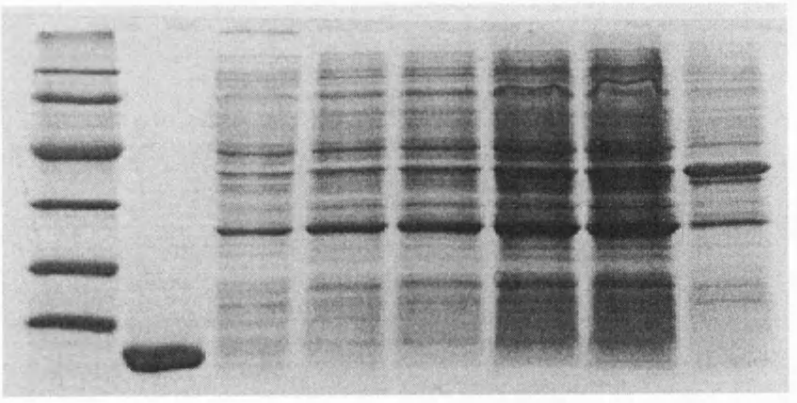
Figure 20. Full length nucleotide sequence for gpRANTES protein (*top*). The coding region begins at position 20 and ends at position 292 and is underlined. Residues 20-43 are a presumed signal sequence. This sequence has been submitted to Genbank under the accession code U77037. Amino acid sequence alignment for guinea pig RANTES with human and murine sequences (*below*), showing presumed leader sequence (-23 to 1) and secreted proteins (1-68). Underlined amino acids highlight the differences in composition between guinea pig and human RANTES.

4.2. Protein expression and purification

Initial attempts to express the guinea pig RANTES protein in *E. coli* using the gpRANTES cDNA proved unsuccessful. No detectable expression of an 8-10 kDa protein was observed with or without the N terminal hexapeptide sequence either in the cell pellet or supernatant following cell breakage (figure 21). However, extremely high levels of expression were obtained when the human RANTES construct carrying the hexapeptide at the amino terminus underwent base substitution. Mutations were made at the following positions numbered according to the sequence in figure 20; 98(T→G), 134(G→T), 140(C→G), 152(G→A), 182(G→A), 197(C→T), 198(C→T), which resulted in the substitution of the 6 amino acids responsible for the difference in primary sequence of the two species (figure 20). Mutations were performed by B. Allet, Glaxo IMB, Geneva. All protein purification was performed by myself at Glaxo IMB.

The purification of gpRANTES was very similar to that described by Proudfoot *et al* (1995) for human RANTES, although where differences arose, details are outlined below. The guinea pig RANTES fusion protein was expressed in inclusion bodies (see figure 25A, lanes 2-4) which were separated from the remaining *E. coli* proteins by size exclusion gel filtration (Sephacryl HR200) after solubilisation in guanidium HCl (figures 22A and 25A, lane 5). Fractions containing proteins between 5-20 kDa on SDS-PAGE were pooled, renatured and concentrated by cation exchange chromatography using a HL26/10 SP column. The major protein peak eluted between 1.3 M and 1.6 M sodium chloride (figure 22B). It is unlikely that any non-renatured protein was carried over at this stage since this is generally insoluble and hence removed by centrifugation prior to separation. If any non-renatured protein remains soluble, this would elute at a different salt concentration.

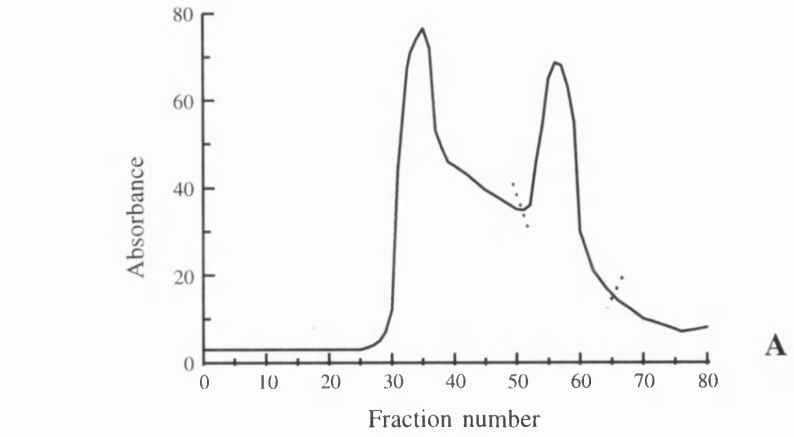
Endoproteinase Arg-C digestion was used to remove the fusion peptide from both human and guinea pig RANTES proteins. In the case of human RANTES, the products were easily separated using cation exchange chromatography. SDS-PAGE analysis of fractions from this column (figure 23) show that most of the product was cleaved hRANTES, running at a lower molecular weight compared to a hexapeptide-hRANTES standard. In comparison, Arg C cleaved the hexapeptide guinea pig protein at more than the required site (25A, lane 6). The cleaved product underwent initial separation by cation exchange chromatography as above with the addition of 6 M urea in the buffers. Under these conditions, cleaved gpRANTES eluted at 0.5 M NaCl, preceding uncleaved gpRANTES at 0.7 M NaCl (figure 22D). However, the mature full length guinea pig protein was isolated from selected fractions by reverse phase HPLC (figure 24, peak b) giving a yield of 0.1 mg per mg fusion protein, and 0.2 mg/g *E. coli* cells. In comparison the yield of huamn RANTES was higher at 0.8 mg/g cells, which may reflect differences in the ease of hexapeptide separation following Arg C cleavage. However, reverse phase HPLC purification for gpRANTES resulted in a single band on SDS-PAGE analysis under denaturing conditions (figure 25A, lane 7). The authenticity of this final product was verified by amino acid composition of the amino terminal (SPYASD) by Edman degradation which was performed by E. Magnenat, Glaxo IMB, Geneva. In addition, electrospray ionisation mass spectroscopy analysis gave a mass of 7897Da which corresponds with the expected mass of 7901Da calculated from the amino acid sequence, with the formation of two disulphide bonds. The final product contained no detectable levels of endotoxin. Bioassay of fractions during the different purification steps indicated that renatured gpRANTES was inactive before removal of the hexapeptide leader, whilst mature full length gpRANTES mobilised $[Ca^{2+}]_i$ in THP-1 cells (figure 25B).

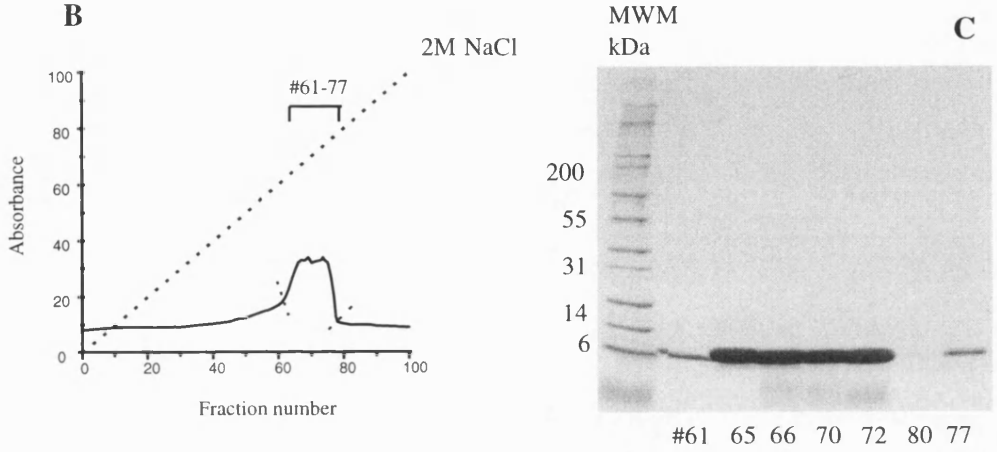


1 2 3 4 5 6 7 8

Lane 1 Molecular weight markers
Lane 2 10μg rhRANTES
Lane 3 non-induced total cells
Lane 4 1/20 dilution of cell break supernatant
Lane 5 1/20 dilution of induced total cells
Lane 6 1/10 dilution of cell break supernatant
Lane 7 1/10 dilution of induced total cells
Lane 8 1/5 dilution of cell break pellet

Figure 21. SDS-PAGE analysis of cell pellet and supernatant following cell breakage of *E. coli* transfected with gpRANTES cDNA. No detectable expression of a protein running at approximately 8-10 kD (compare with rhRANTES standard in lane 2) was observed in either cell pellet or supernatant.





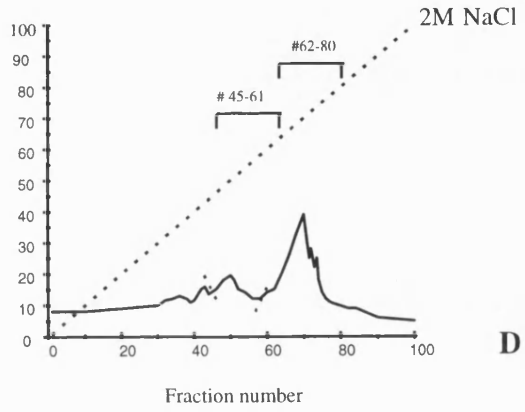
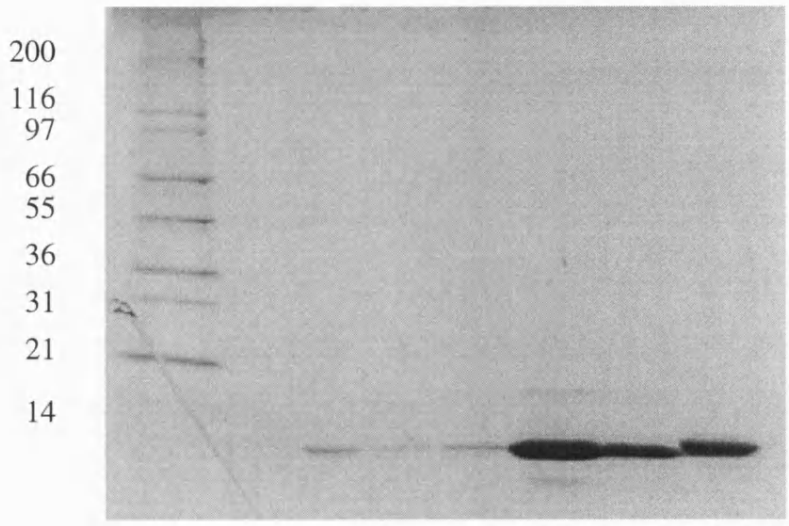


Figure 22. Purification steps of gpRANTES from *E. coli*. (A) Separation from inclusion body components using size exclusion gel filtration (Sephacryl HR 200) under denaturing conditions. Fractions 52 to 65 containing 5-20 kD protein were pooled, renatured and concentrated by cation exchange chromatography (figure B and SDS-PAGE of fractions in C). Fractions 61-77 containing approx 8 kD protein eluting between 1.3-1.6 M NaCl (B), were pooled and digested with Arg C. Digested products were initially separated by cation exchange chromatography (D). Most cleaved protein eluted within fractions 45-61 (0.5 M NaCl), preceding uncleaved gpRANTES (#62-80).



Lane 1	Molecular weight markers (kDa)
Lane 2-6	Predominant peak HPLC products from cation exchange column fractions
Lane 7	rhRANTES*
Lane 8	hexapeptide hRANTES*

Figure 23. SDS-PAGE analysis of fractions from cation exchange chromatography resulting from the separation of Arg C digestion of hexapeptide-human RANTES. Digestion products were separated by applying to a HiLoad S (26/10) column equilibrated with 50 mM sodium acetate pH 4.5 and eluting with a linear gradient of NaCl. Fractions from this column (lanes 2-6) contained cleaved hRANTES which ran at a lower molecular weight than the uncleaved protein (lane 8). Lane 1; molecular weight markers, lane 7; cleaved rhRANTES. *Purified standards.

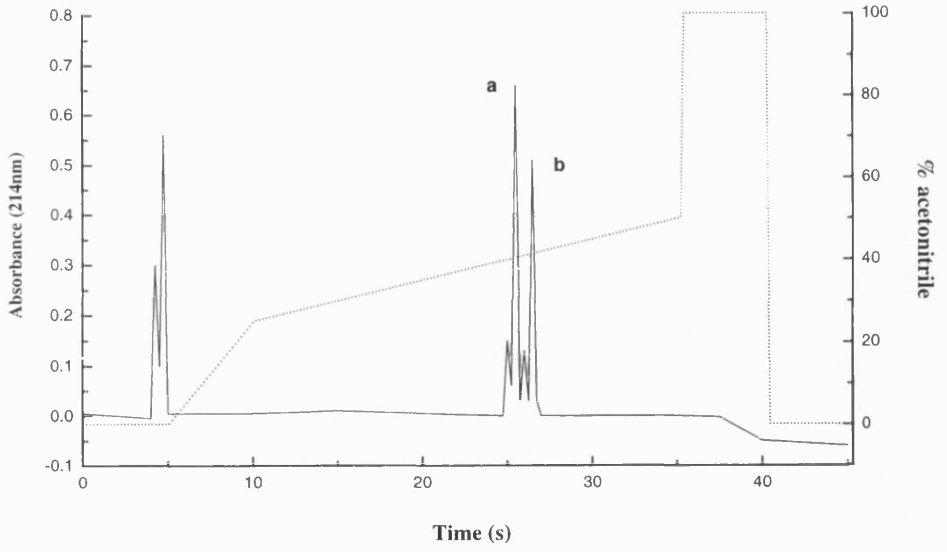


Figure 24. Reverse-Phase HPLC trace to show separation of Arg C-digested hexapeptide-gpRANTES. Peak **a** represents the uncleaved fusion protein, peak **b** represents gpRANTES (cleaved) as confirmed by N terminal sequencing (**b**=SPYAS) and mass spectroscopy (**a**=8869 Da, **b**=7897 Da).

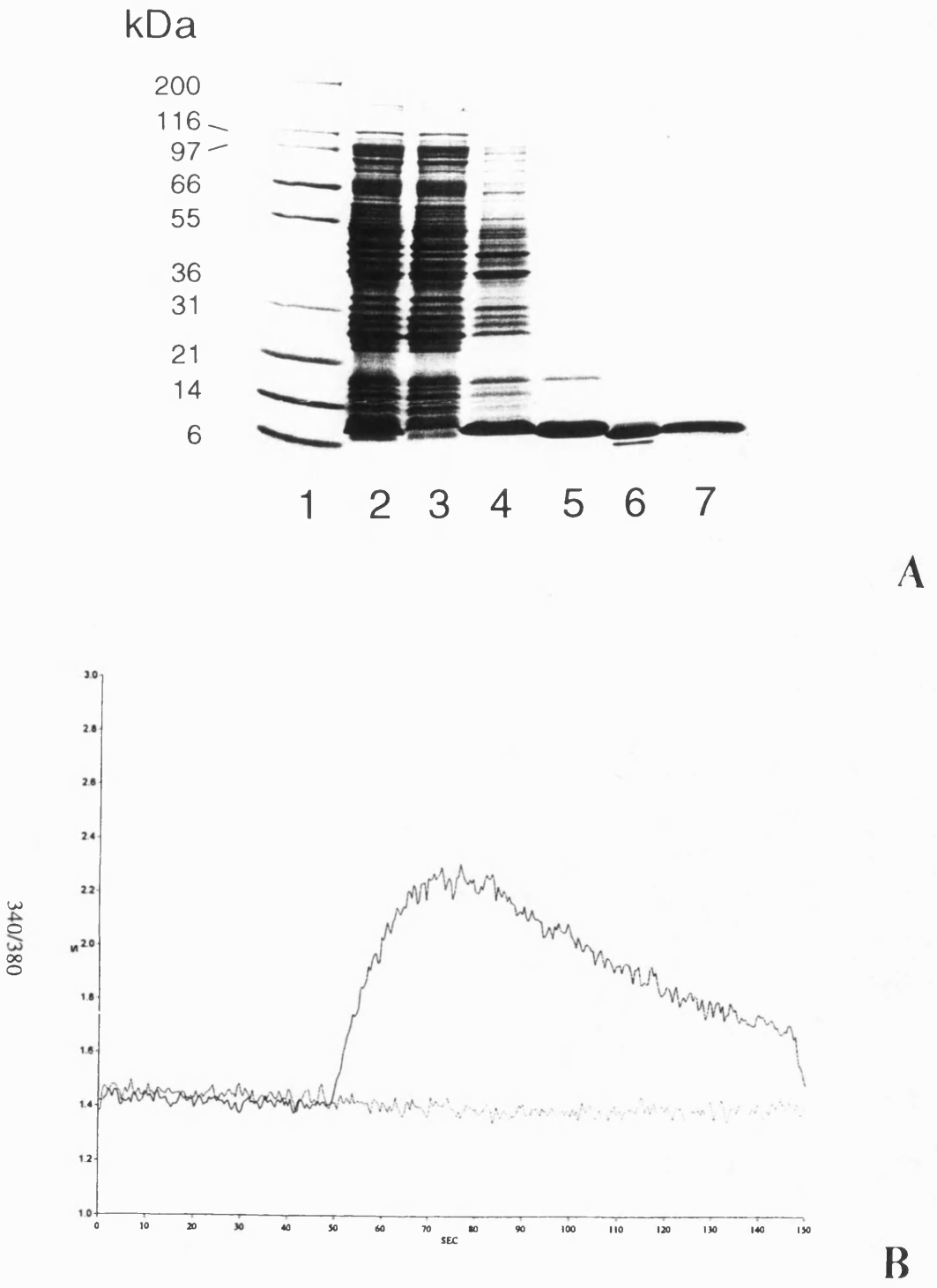


Figure 25. (A) SDS-PAGE analysis of the purification of gpRANTES protein from *E. coli*. Lane 1 shows molecular weight markers, lane 2; total *E. coli* products, lane 3; supernatant from *E. coli* extraction, lane 4; *E. coli* inclusion body, lane 5; size exclusion gel filtration pool (Sephacryl HR 200), lane 6; products following Arg C digestion, lane 7; recombinant gpRANTES. (B) Recombinant gpRANTES was able to induce changes in $[Ca^{2+}]_i$ in monocytic THP-1 cells whilst a similar concentration of fusion-protein-gpRANTES was completely inactive (below). This assay was used as a routine 'screen' at each step following the Arg C cleavage process to identify fractions containing cleaved gpRANTES.

4.3. *Attempts to identify RANTES in guinea pig platelets*

Guinea pig RANTES could not be expressed in *E. coli* using the gpRANTES cDNA (figure 21). Mutation of a human RANTES construct, as described above, yielded high quantities of gpRANTES but with the same N-terminal start point as hRANTES. The identification of a native form of guinea pig RANTES would allow confirmation of the N terminal sequence and since thrombin-stimulated human platelets are a source of RANTES (Kameyoshi *et al.* 1992), guinea pig platelets were examined for the possible presence of the chemokine.

Guinea pig platelets were isolated from acid-citrated whole blood and stimulated with 2U/ml thrombin under similar conditions as described for human platelets (Kameyoshi *et al.* 1992). Thrombin induced a marked aggregation of the platelets, as assessed by a increase in light transmission using an aggregometer (figure 26). Over n=4 platelet preparations from separate animals, this dose of thrombin induced a mean increase in the light transmission of 76 %.

Thrombin-stimulated platelet supernatants were assayed for their ability to increase $[Ca^{2+}]_i$ in guinea pig peritoneal macrophages. Results in chapter 5 of this thesis demonstrate the ability of RANTES to activate these cells. The supernatant did induced changes in $[Ca^{2+}]_i$ in these cells whilst an equivalent carry-over amount of thrombin (0.1U/ml) did not. However this effect was completely inhibited by 1 μM of the PAF antagonist, WEB 2086. This dose of WEB 2086 completely inhibited the increase in $[Ca^{2+}]_i$ induced by 100 nM PAF in these cells (figure 27). Thus no 'RANTES-like' activity was detected in guinea pig thrombin-stimulated platelet supernatants.

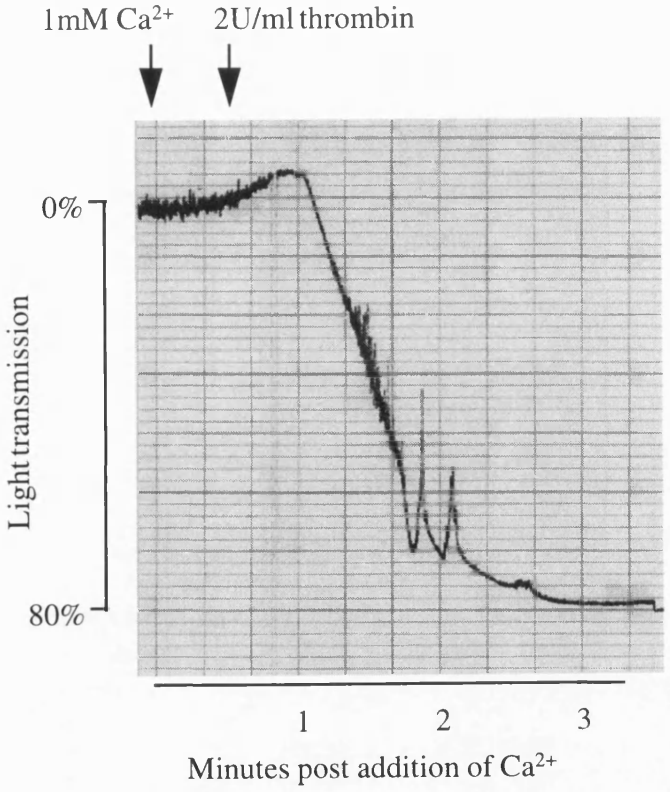
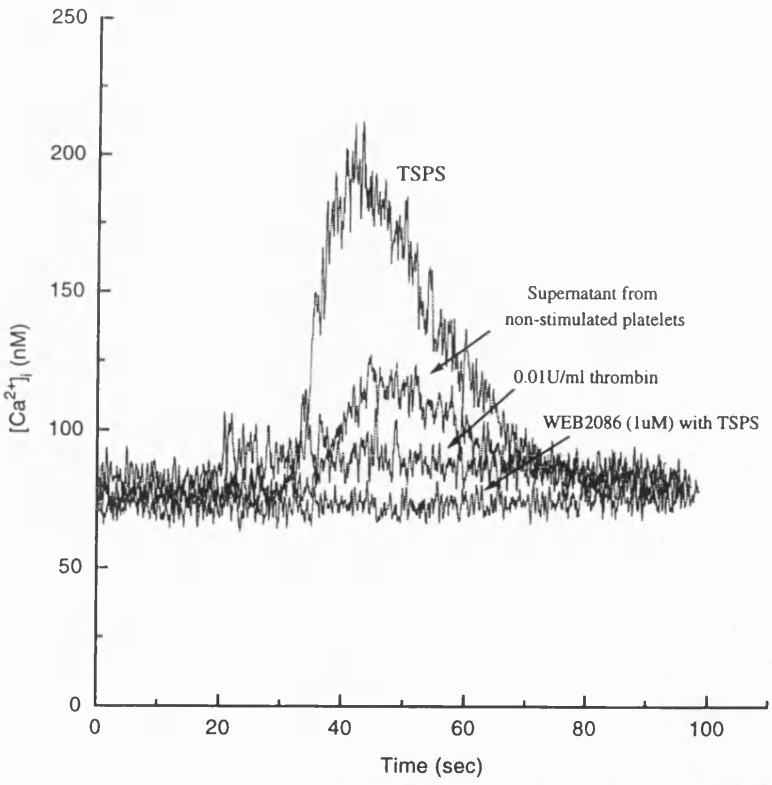


Figure 26. An example trace of platelet aggregation induced by thrombin (2U/ml). Aggregation of platelets at an initial density of $2x10^8$/ml was assessed by increased light transmission. The mean change in light transmission using n=4 platelet preparations from separate animals was 76±5 %.



Treatment of gp peritoneal MØ	Mean change in $[Ca^{2+}]_i$ ±SEM nM
TSPS	112.3±15
TSPS + 1 μM WEB 2086	5.5±1.6
0.1 U/ml thrombin	4.1±1.8
100 nM PAF	343.8±98.2
100 nM PAF + 1 μM WEB 2086	17.8±7.2

Figure 27. *Above* Representative trace to show increases in $[Ca^{2+}]_i$ observed following addition of thrombin-stimulated platelet supernatant (TSPS) to fura-2 loaded guinea pig peritoneal macrophages ($1x10^6$/ml). The effect was inhibited by pre-addition of 1 μM WEB 2086. *Below*. Mean±SEM changes in $[Ca^{2+}]_i$ following addition of TSPS with or without WEB 2086. Responses are from macrophage preparations from *n*=3 animals and using TSPS prepared from platelets from different guinea pigs.

4.4. Summary of results: expression and purification of recombinant gpRANTES

- gpRANTES is highly homologous to hRANTES at both the nucleotide (90%) and amino acid level (87%).

- gpRANTES protein would not express in *E. coli* using the gpRANTES cDNA. However, high levels of expression were obtained when the human RANTES construct encoding a hexapeptide tail was mutated to obtain the substitution of the 6 amino acids responsible for the difference in primary sequence of the two species.

- The gpRANTES-hexapeptide protein was expressed in the inclusion body of *E. coli*. Following cleavage to remove the hexapeptide sequence, full length mature gpRANTES was purified to single-peak RP-HPLC purity and was able to induce changes in $[Ca^{2+}]_i$ in THP-1 cells.

- No evidence was observed for the presence of RANTES in thrombin-stimulated platelet supernatant. The supernatant was able to increase $[Ca^{2+}]_i$ in guinea pig peritoneal macrophages but this was completely inhibited in the presence of the PAF antagonist, WEB 2086.

CHAPTER 5: RESULTS:

Biological characterisation of recombinant gpRANTES

5.1. In vitro biological activity

Guinea pig peritoneal eosinophils and macrophages and human peripheral blood eosinophils were used to assess the biological activity of gpRANTES. The efficiency of cell isolation procedures were confirmed by histological analysis of Diff-Quik stained cytospins (figure 28). The purity and viability of isolates was >93 %.

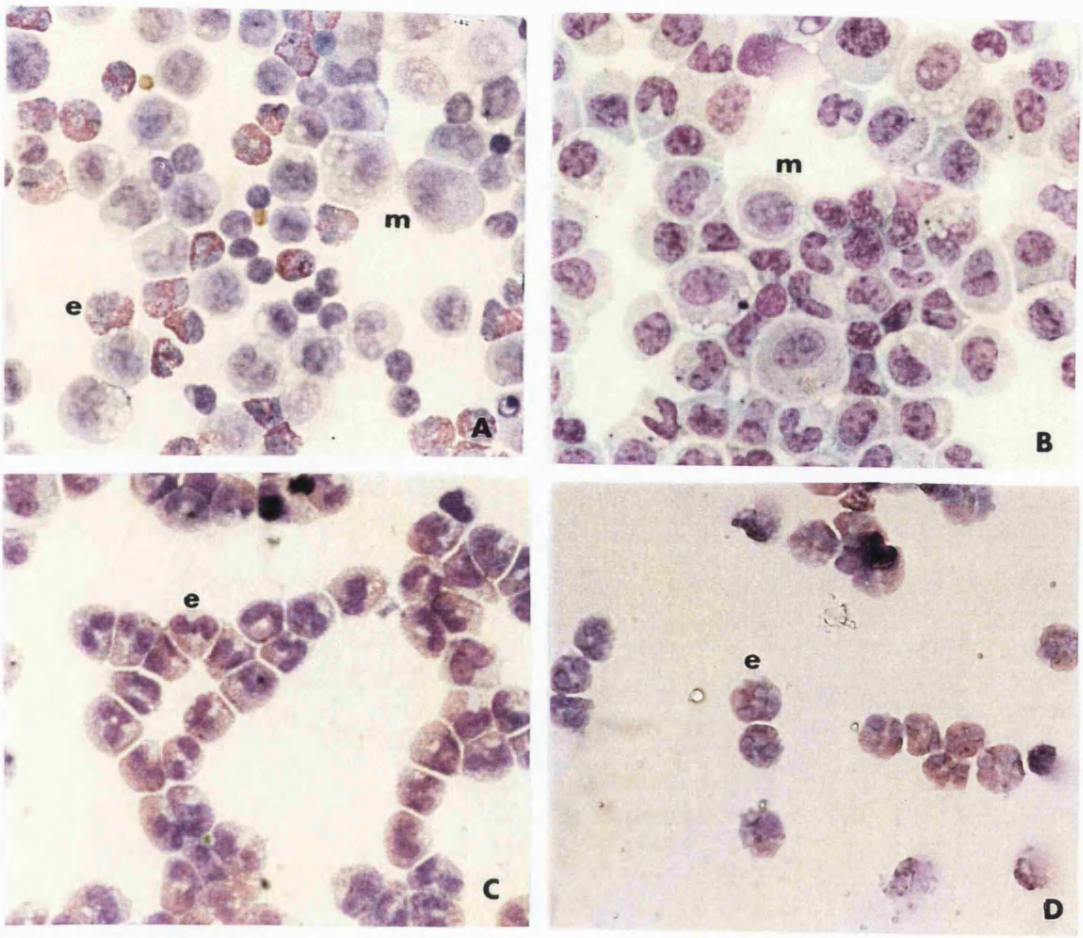


Figure 28. Representative cytospins of guinea pig peritoneal lavage cells (A), peritoneal macrophages (B) and eosinophils (C) and human peripheral blood eosinophil (D) isolations. Contaminating cell types were no greater than 4 % of the total cells counted. m = macrophage, e = eosinophil, x1000.

Guinea pig peritoneal eosinophils were initially examined for their ability to migrate in response to recombinant human C5a. C5a induced a bell-shaped response with maximal response at 30 nM. Figure 29 shows the migratory response in n=3 different eosinophil preparations. Since C5a was always an effective chemotactic agent, it was routinely used as a positive control in the experiments examining the migratory effects of the chemokines. However, the extent of the response to C5a varied between different sets of experiments.

Guinea pig RANTES was able to induce a pronounced chemotactic response in human eosinophils with similar potency to hRANTES. Both proteins caused a maximal response at 30 nM but higher concentrations caused a decrease in the migratory response (figure 30). However, neither protein was able to stimulate a significant chemotactic response of guinea pig eosinophils, although cells were responsive to C5a.

It has previously been shown that IL-3 and GMCSF-primed guinea pig eosinophils are able to respond to the CXC chemokine IL-8 (Burrows *et al.* 1991) and thus it was important to determine whether primed guinea pig eosinophils might be RANTES-responsive. In order to determine a suitable concentration of a priming cytokine, guinea pig eosinophil chemotactic responses to human recombinant IL-5, IL-3 and GMCSF were assessed (figure 31). IL-3 and GMCSF were unable to induce the migration of these cells at the concentrations tested (1-100 nM). In comparison, IL-5 caused a slight concentration-dependent increase in migration, inducing the chemotaxis of 35±8 eosinophils compared to a basal value of 4±2 eosinophils. In an attempt to unveil a RANTES response to IL-5, a threshold concentration of this cytokine (ie. a concentration at which the cytokine alone did not induce eosinophil chemotaxis), 3 nM, was tested together with gpRANTES. As can be seen in figure 32, guinea pig

eosinophils did not migrate over vehicle basal levels in response to IL-5 alone, RANTES alone, or RANTES plus IL-5. This was in contrast to the CXC chemokine IL-8, which alone appeared to cause a very slight chemotaxis of guinea pig peritoneal eosinophils. This response was substantially potentiated in the presence of IL-5. The lack of response of guinea pig eosinophils to RANTES in the chemotaxis bioassay was mirrored by an inability to increase $[Ca^{2+}]_i$. The maximal response of $[Ca^{2+}]_i$ observed with either gpRANTES or hRANTES was negligible in guinea pig eosinophils. Conversely, gpRANTES caused a rapid increase in $[Ca^{2+}]_i$ in human eosinophils (figure 33). In marked contrast, guinea pig peritoneal macrophages were strongly activated by gpRANTES, as assessed by changes in $[Ca^{2+}]_i$ (figure 34A) and chemotaxis (figure 34B). In the same sets of macrophages, hRANTES demonstrated similar levels of potency. An increase of 321±56 nM in $[Ca^{2+}]_i$ was observed following addition of 100 nM hRANTES and 61±5 nM $[Ca^{2+}]_i$ following addition of 10 nM.

Guinea pig peritoneal macrophages were also activated using recombinant human MIP-1α. rhMIP-1α induced a dose dependent increase in $[Ca^{2+}]_i$ in these cells as is shown in figure 35 (n=3 animals). rhMIP-1β also activated these cells but was slightly less potent at 100 nM.

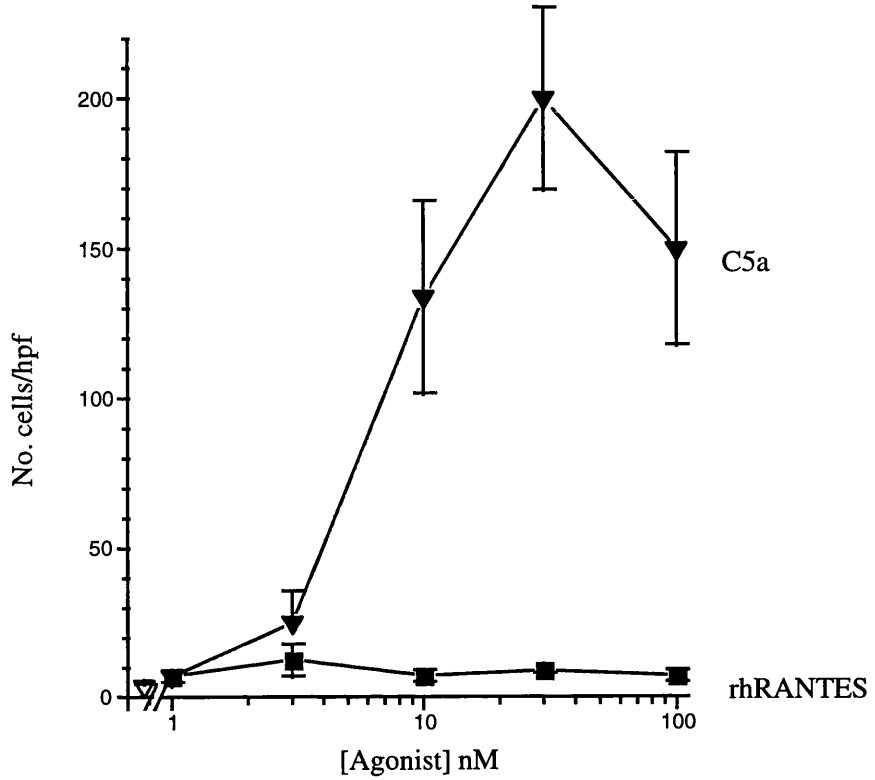


Figure 29. Chemotactic response of guinea pig eosinophils induced by recombinant human C5a as assessed by cells migrated per high power field (x400). The response of these cells towards hRANTES is also shown. Values represent the mean±SEM using n=4 eosinophil preparations from different donor animals.

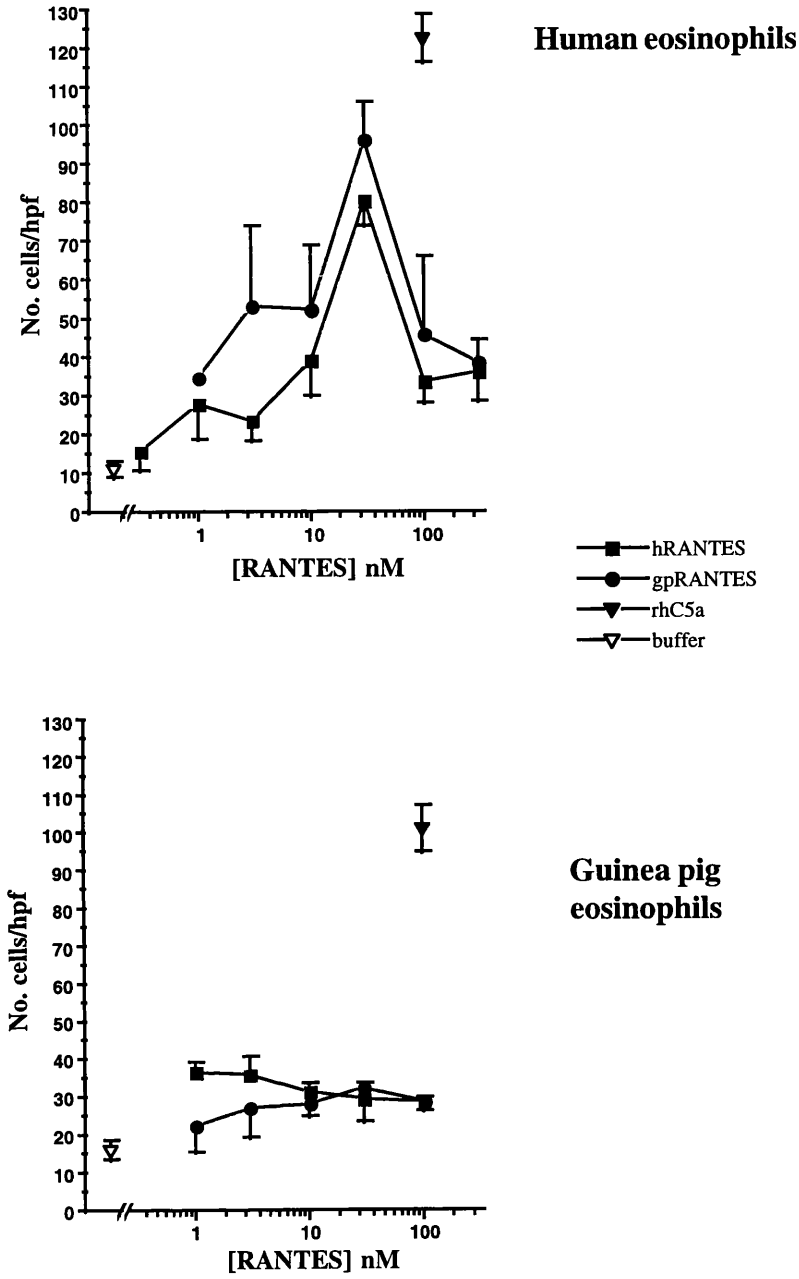


Figure 30 *In vitro* chemotaxis towards gpRANTES and hRANTES of human peripheral blood eosinophils (*top*) and guinea pig peritoneal eosinophils (*bottom*), as assessed by cells migrated per high power field (x400). Responses are compared to that for 100 nM rhC5a and basal migration to buffer. In each experiment 4 replicates of each test sample dilution were performed. Results are expressed as mean±SEM for n=3 eosinophil preparations from different donors.

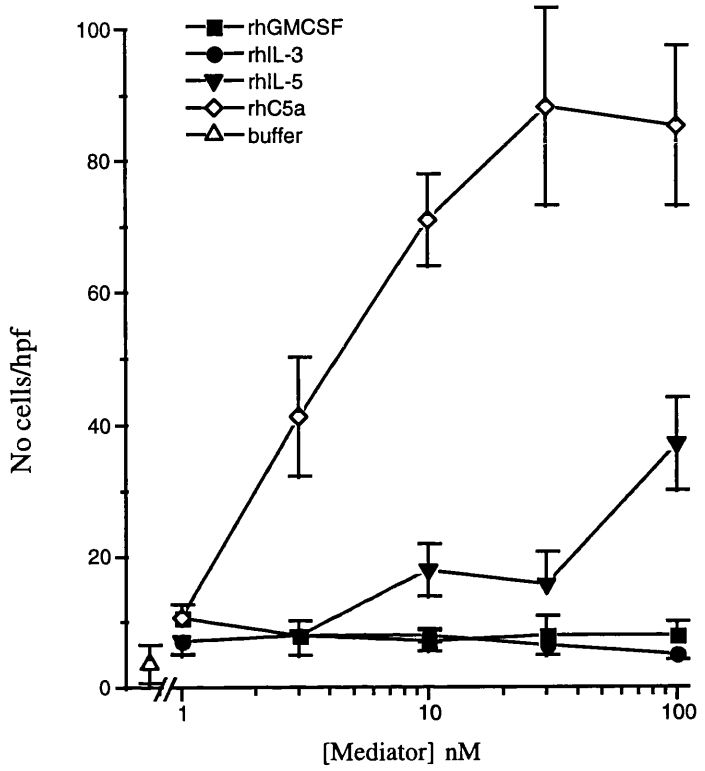


Figure 31. Chemotactic responses of guinea pig peritoneal eosinophils in response to IL-5, IL-3 and GMCSF with rhC5a as positive control. A slight migratory response was noted using IL-5 but not IL-3 or GMCSF. 3 nM IL-5 was selected as a priming dose for subsequent experiments since this concentration alone did not induce the migration of these cells. Points represent mean±SEM for n=3 eosinophil preparations from different animals. Chemotactic response was assessed by the number of cells migrated per high power field (x400).

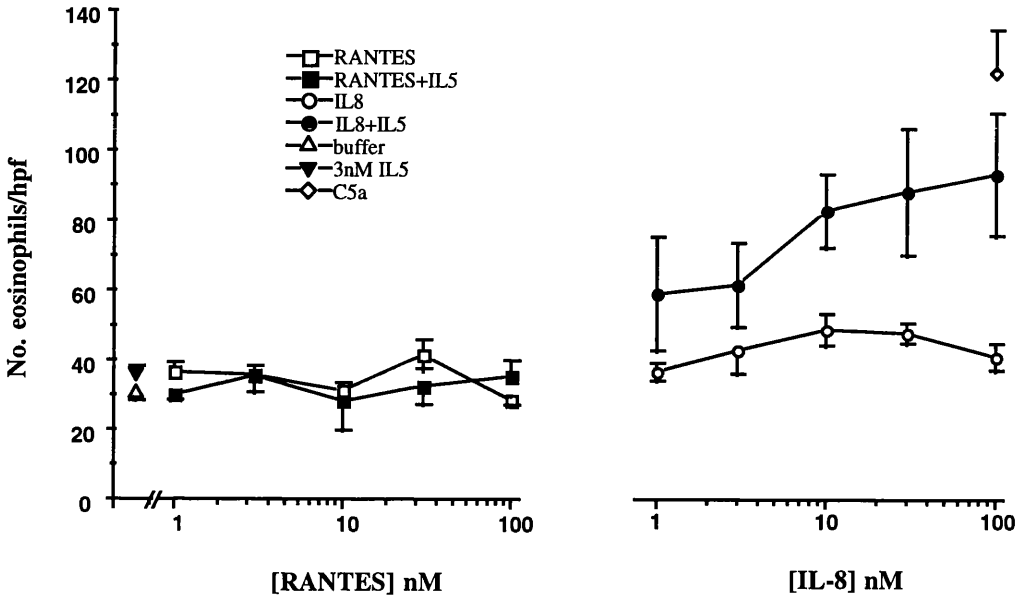


Figure 32. Chemotactic response of guinea pig eosinophils towards RANTES (*left*) and rhIL-8 (*right*) in the presence and absence of 3 nM IL-5, 100 nM rhC5a and vehicle migration or to 3 nM IL-5 alone. In each experiment 4 replicates of each test sample were performed. The chemotactic response was assessed by cells migrated per high power field (x400). Points represent mean±SEM for n=3 eosinophil preparations from different animals.

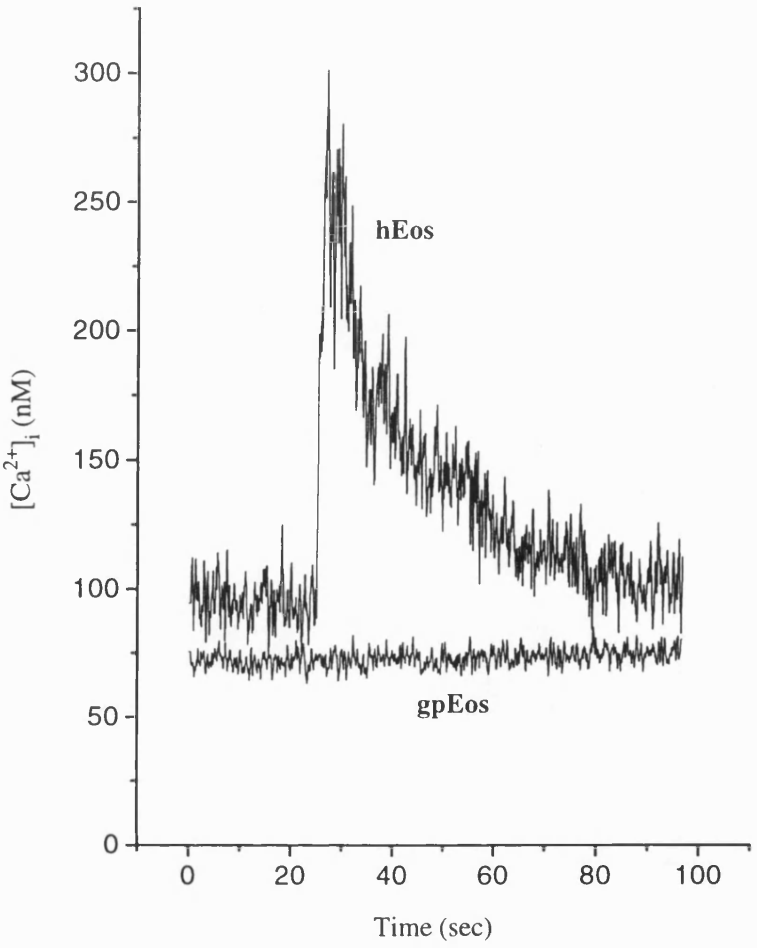


Figure 33. Representative time course trace to show the effect of 100 nM gpRANTES addition at time=22 s on $[Ca^{2+}]_i$ in human and guinea pig eosinophils. The mean peak change in $[Ca^{2+}]_i$ ±SEM in eosinophils following addition of 100 nM gpRANTES for *n*=3 different human donors was 121±40nM compared to 10±4nM for eosinophils from *n*=3 separate guinea pigs.

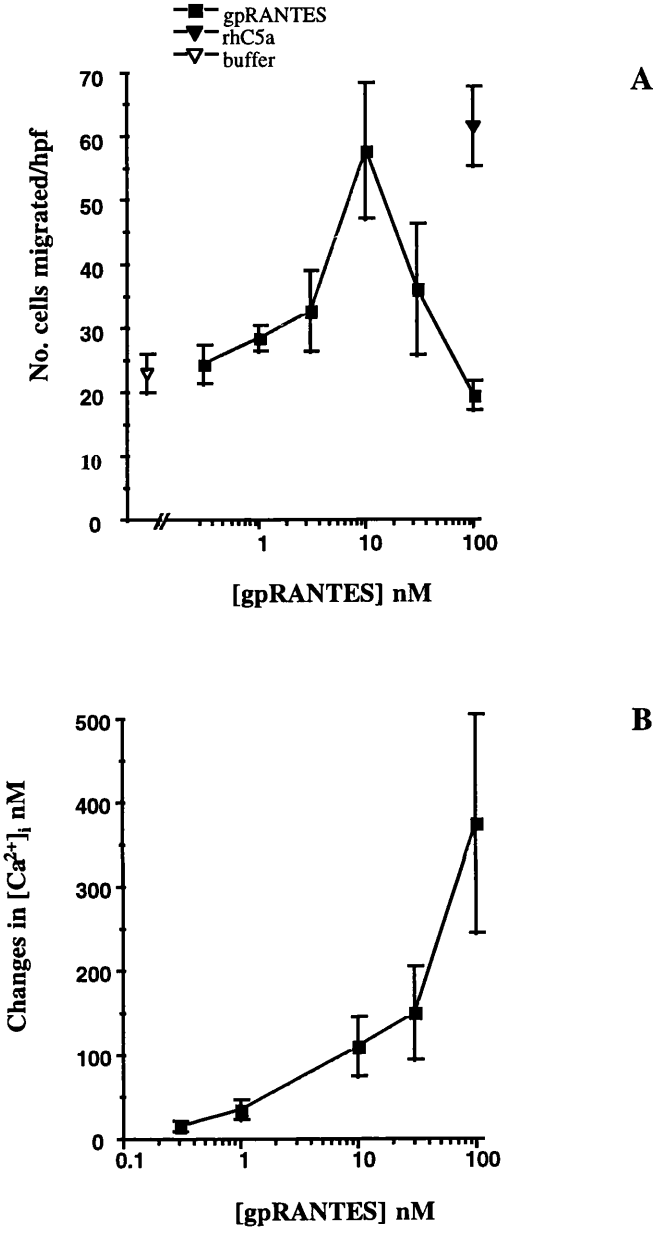


Figure 34. Dose dependent effect of gpRANTES on the migration (A) and on changes in $[Ca^{2+}]_i$ (B) in guinea pig peritoneal macrophages. Points represent mean±SEM for n=3 macrophage preparations from different animals. The chemotactic response was assessed by cells migrated per high power field (x400).

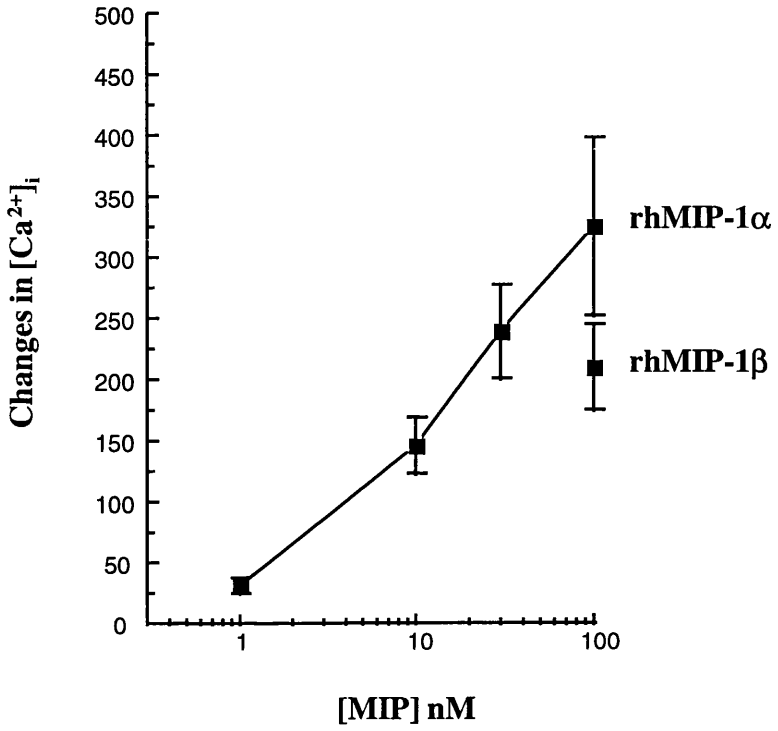


Figure 35. Dose dependent changes in $[Ca^{2+}]_i$ in guinea pig peritoneal macrophages induced by rhMIP-1α. Data represents mean peak ± SEM using cells from *n*=3 separate guinea pigs. Using the same cell preparations 100 nM rhMIP-1β induced peak changes in $[Ca^{2+}]_i$ of 210±35 (mean ± SEM). Responses represent mean±SEM for *n*=3 macrophage preparations from different animals.

Generation of hydrogen peroxide in guinea pig macrophages.

Guinea pig macrophages were analysed for their ability to generate hydrogen peroxide in response to human and gpRANTES. Table 4.2 summarises the results of hydrogen peroxide generation in two sets of macrophages from different guinea pigs. Since only a minimal response was observed using either human or guinea pig RANTES compared to the positive control, C5a, this experiment was not repeated.

[Agonist] nM	Experiment 1	Experiment 2
gpRANTES 3 nM	1.07±0.05	3.28±0.16
gpRANTES 10 nM	1.57±0.04	3.92±0.06
gpRANTES 30 nM	1.97±0.03	4.54±0.28
gpRANTES 100 nM	2.33±0.19	3.36±0.42
hRANTES 3 nM	-	4.28±0.34
hRANTES 10 nM	2.22±0.11	4.76±1.06
hRANTES 30 nM	-	5.70±1.23
hRANTES 100	2.48±0.04	4.14±0.62
rhC5a 100	5.20±0.09	7.80±1.23
Basal (vehicle)	0.9±0.07	3.01±0.75

Table 5.1 Hydrogen peroxide generation from two guinea pig macrophage preparations using different donor animals. Values are expressed as mean±SEM from n=4 wells per agonist dilution.

Responses of blood derived eosinophils in vitro

Guinea pig whole blood from was collected from animals 1 h after injection with 18.3 pmol/kg rhIL-5. Blood eosinophilia has previously been shown to be maximal at this time point following injection of the same dose of rhIL-5 in the guinea pig (Collins *et al.* 1995). Citrated blood was separated using dextran and upper layer centrifuged to pellet the cells. These cells were then separated on a discontinuos Percoll gradient as described for peritoneal cells. However, this separation step was not found to be effective and most cells were banded between a density of 1.080-1.085 g/ml. Cytospins of this band revealed that 35±7 % were mononuclear cells, 39±9 % were neutrophils, 18±9 % were bilobed cells (presumably eosinophils but with weak granular staining) and 7±3 % were eosinophils with intense granular staining (mean±SEM for n=4 leukocyte preparations from different animals).

The mixed cell population from the Percoll gradients was analysed for an ability to migrate toward gpRANTES using the microchemotaxis chamber. At the concentrations of gpRANTES tested (1-100 nM), no significant differences in the number of migrated cells were count compared to saline/BSA control wells. In comparison, 100 nM rhC5a induced a significant increase ($p<0.01$) in the number of cells that migrated compared to controls using cells from n=4 different animals. Examination of the filter following chemotaxis revealed that the migrated cells were predominantly neutrophils in C5a-treated wells.

These results are detailed in table 5.2. Raw data was compared to control (basal) using two-way ANOVA followed by Dunnett's test. Since basal migration varied considerably between each experiment, results are expressed as chemotactic index (CI)

which is calculated by dividing the mean number of cells migrated to a given concentration of gpRANTES by the mean basal migration to saline/BSA.

Mediator	nM	Chemotactic index
gpRANTES	1 3 10 30 100	1.02±0.2 1.09±0.1 1.41±0.2 1.63±0.6 2.06±0.7
hRANTES	10 100	1.74±0.3 1.67±0.3
rhC5a	100	2.97±0.8 *p<0.01
Saline/BSA (basal)	-	136.8±47.4 (cell nos.)

Table 5.2. Summary of chemotactic responses of blood leukocytes to gpRANTES and rhC5a. No significant migration was observed for gpRANTES compared to basal migration. In comparison, C5a induced a significant increase in cells migrated over basal levels. Results are using blood cells from n=4 preparations from different animals. Chemotaxis was assessed by number of cells migrated per high power field (x400). However, results are assessed as chemotactic index except for basal migration.
* Significant difference ($p<0.01$) in comparison to basal migration using two-way ANOVA with Dunnett's test on raw data.

5.2. In vivo macrophage recruitment in the guinea pig

5.2.1. Effects of tracheal instillation of gpRANTES

Cellular recruitment into the lung following tracheal instillation of gpRANTES was assessed by changes in total BAL cell numbers and differential cell counts. gpRANTES caused a dose dependent increase in the total cells in the BAL 24 h after instillation. This effect was predominantly due to increased numbers of macrophages, with no detectable increase in recruitment of eosinophils, neutrophils or T lymphocytes (figures 36, 37A). Time course studies revealed that an increase in cell numbers was observed as early as 6 h and was maintained until at least 48 h (B). The predominant cell type at all the time points was the macrophage, although at 48 h a small increase in eosinophils and neutrophils was evident.

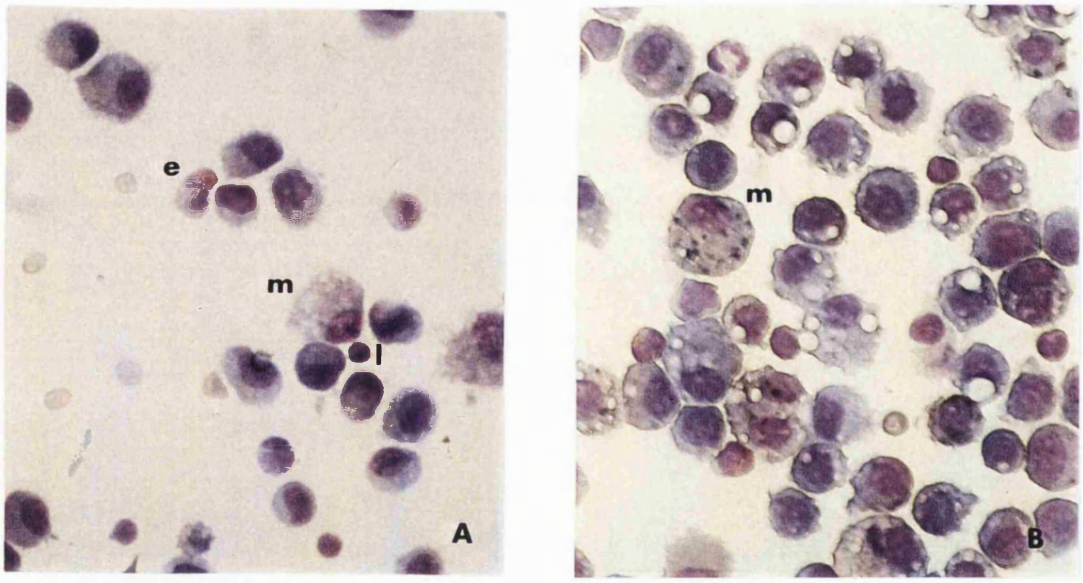


Figure 36. Representative cytospins of BAL cells from guinea pigs at 24 h following tracheal instillation of either saline (A) or 10 μg gpRANTES (B). In all cases, lungs were lavaged with the same volume of buffer. m=macrophage, l=lymphocyte, e=eosinophil

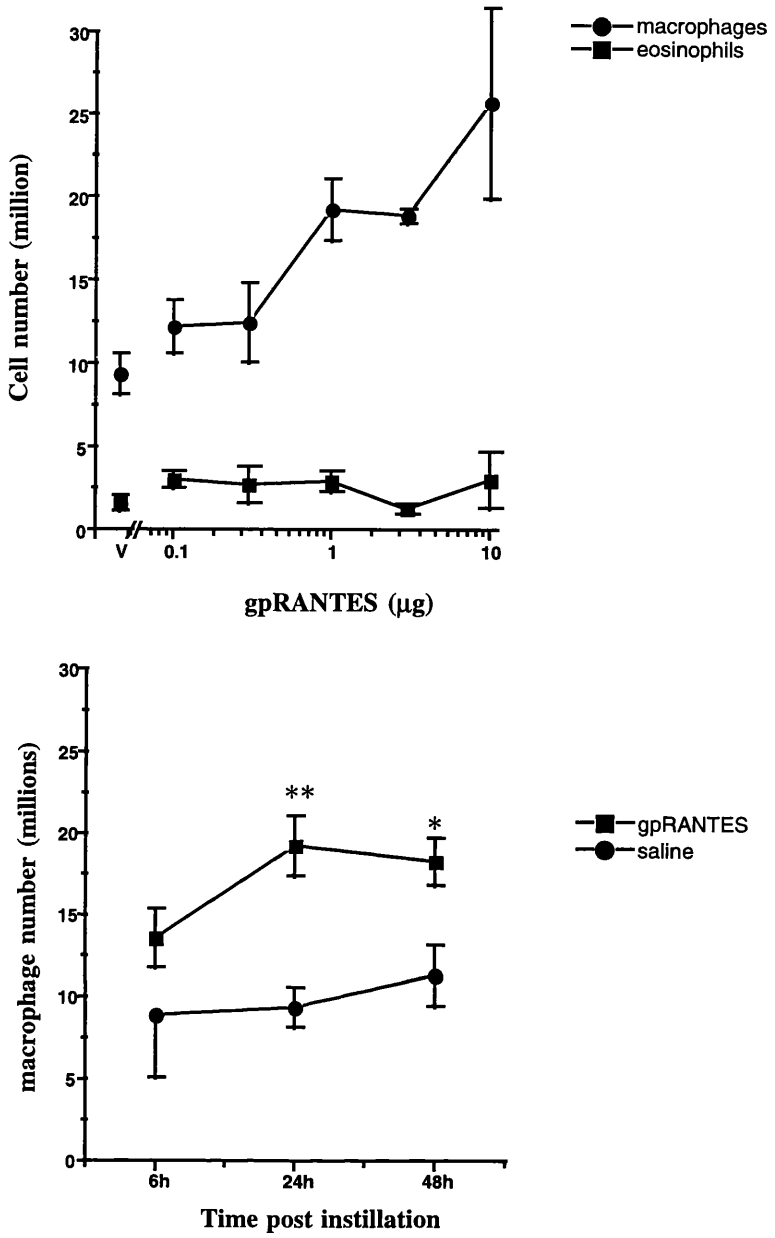


Figure 37. Tracheal instillation of gpRANTES induced a dose and time-dependent increase in number of BAL MØ but not eosinophils. (A) Dose-dependent increases in total BAL MØ number. Results are mean±SEM cell number at 24 h for n=3 animals (0.1, 0.3, 3, and 10 μg) and for n=9 (1 μg and vehicle control V). Lymphocyte and neutrophil numbers did not significantly rise above those for control animals (data not shown). *Significant difference ($p<0.05$, ** $p<0.01$) in comparison to control macrophage numbers using ANOVA with post-hoc Dunnett's test. (B) Time dependent increases in MØ number following instillation of 1 μg gpRANTES or vehicle. Results are mean±SEM total macrophage number in BAL for n=3 animals at 6 h, n=6 at 48 h and n=9 at 24 h for both RANTES and saline instilled animals. *Significant difference ($p<0.05$, ** $p<0.01$) in comparison to time-matched control using Student's t test.

5.2.2. Dermal response to injection of gpRANTES

5.2.2.1 ^{111}In eosinophil accumulation in response to chemokines. The leukocyte migratory responses in the guinea pig *in vivo* were assessed using a model as described by Faccoli *et al* (1991), whereby ^{111}In -labelled cells accumulate at skin sites injected with inflammatory mediators. Work done as part of this study demonstrated that ^{111}In-labelled guinea pig peritoneal eosinophils did not accumulate above basal levels in response to rhRANTES, rhMCP-1 or rhIL-8. In comparison, the positive control, guinea pig zymosan activated plasma - a rich source of C5a-des-Arg did induce the accumulation of these cells (figure 38A). Concurrent analysis of ^{125}I albumin accumulation in these skin sites in the same guinea pigs indicated that these chemokines alone did not induce increases in plasma leakage over that observed for vehicle-injected sites. However, plasma leakage was observed using the positive control, histamine, of 87±8.6 µl/site compared to 21±5 µl/site for saline (figure 38B). In these experiments mean±SEM represents responses of ^{111}In-eosinophil accumulation and plasma leakage for n=4 donor eosinophil preparations from different animals with each cell preparation injected into a separate recipient guinea pig.

In further experiments using two different donor with two different recipient guinea pigs, no ^{111}In eosinophil accumulation was observed in sites injected with these chemokines alone or in conjunction with the potent vasodilator, PGE_1 (10^{-10} mol/site; figures 39 and 40). In addition, IL-5 also failed to induce ^{111}In-eosinophil accumulation (figure 39). In these experiments the numbers of ^{111}In-eosinophils per site were 2.5-4 fold less compared to those in figure 38. It is possible that the activation of the donor eosinophils during their purification might cause their sequestration at sites such as the lung, in the recipient animals. In one set of animals (n=2) a slight increase in plasma leakage in sites injected with 10^{-10} mol/site rhRANTES and PGE_1 was observed (figure

39) . The reason for this is not known, however it is possible that this was associated with a RANTES-induced histamine release from basophils as previously reported (Kuna *et al.* 1992) and Alam and colleagues (1992) have demonstrated a similar role for MCP-1. Furthermore, the action of histamine has been demonstrated to be potentiated in the presence of vasodilators such as PGE_1 (Williams and Morley 1973). In work presented for this thesis, plasma leakage was investigated in response to IL-8 and MCP-1 in the presence of 10^{-10} mol/site PGE_1. Plasma leakage was no greater than for PGE_1 alone (figure 40).

This model was not used to further analyse the activity of gpRANTES since *in vitro* data indicated that both gpRANTES and hRANTES were not able to active guinea pig peritoneal eosinophils (figure 30). Since peritoneal cells were used in these *in vivo* experiments, this would account for the inability of hRANTES to induced ^{111}In eosinophil accumulation in this model. Furthermore, a pertinent question at that stage of my work was whether gpRANTES might induce the migration of blood-derived eosinophils ie. not elicited cells.

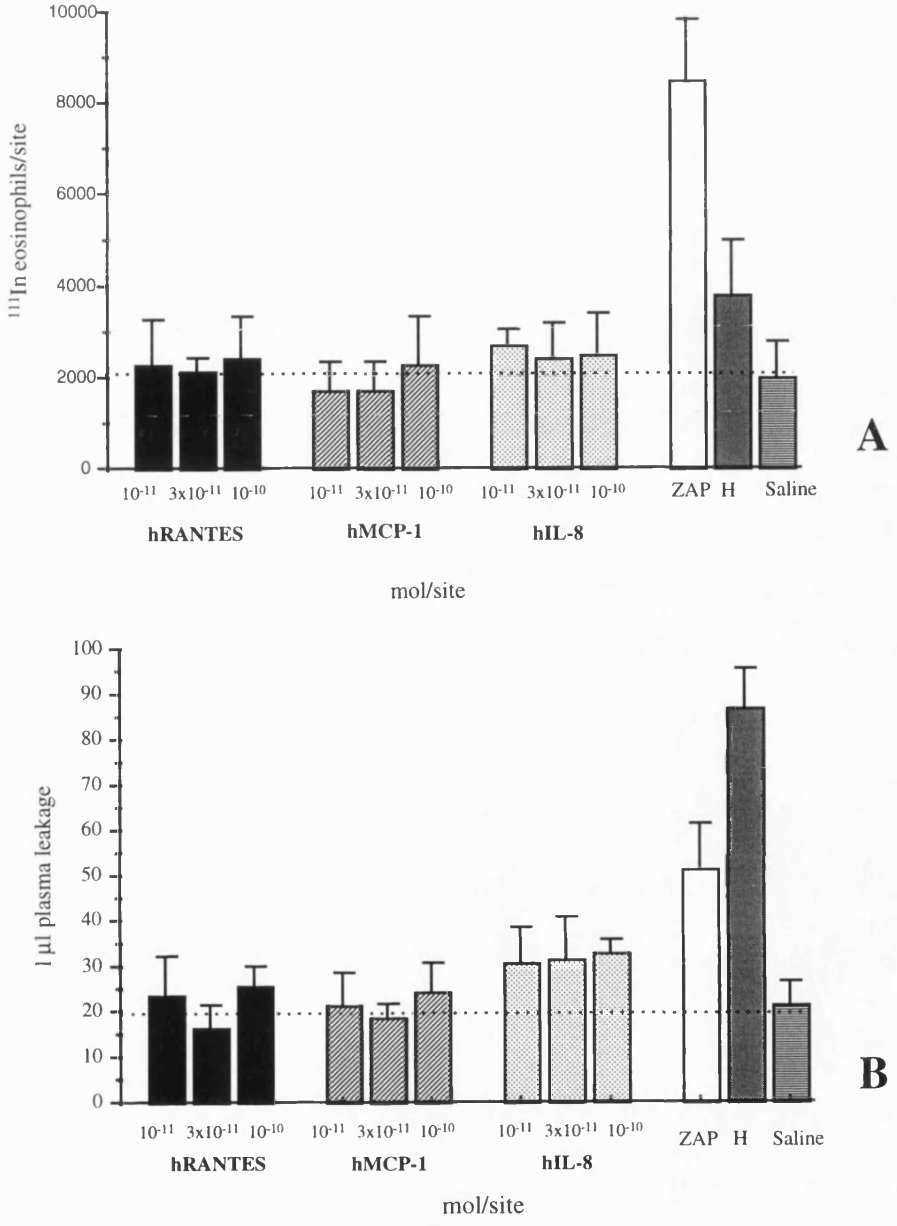


Figure 38 **A**. ^{111}In-labelled eosinophil accumulation in skin sites injected with guinea pig ZAP, but not in response to rhRANTES, rhMCP-1 or rhIL-8. In the same animals, plasma leakage was also monitored by accumulation of ^{125}I albumin (**B**), although increases over saline-injected sites were only observed using histamine. In both figures the dotted line represents mean basal accumulation. Each mediator was injected in duplicate per guinea pig. All bars represent the mean ± SEM of n=4 donor eosinophil preparations in 4 different recipient guinea pigs. Histamine was used at $2.5x10^8$ mol/site and ZAP used as 100 µl of a 1/5 dilution.

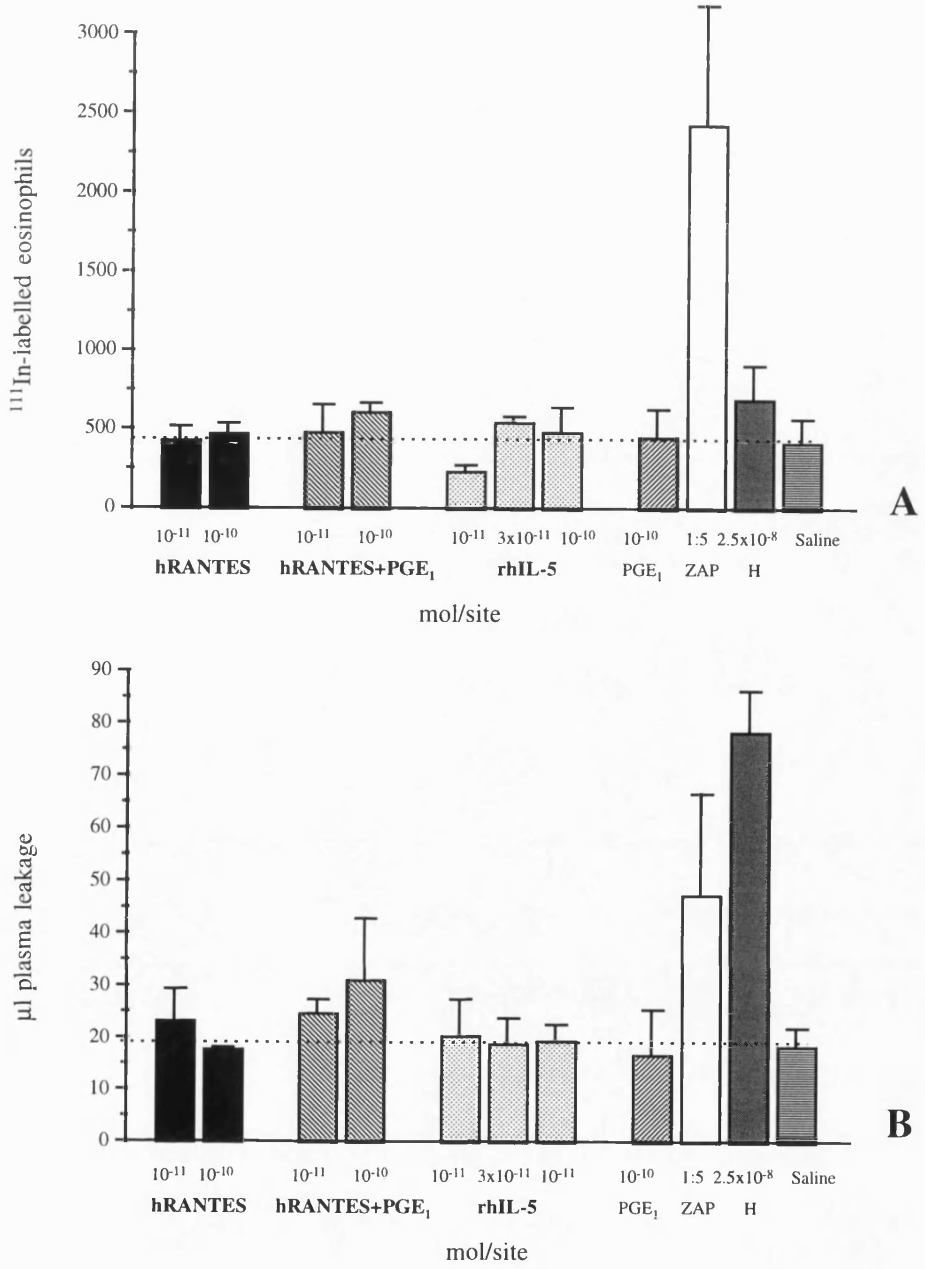


Figure 39 **A**. ^{111}In-labelled eosinophils accumulated in skin sites in injected with guinea pig ZAP, but not in response to rhRANTES +/- PGE_1 (10^{-10} mol/site) or IL-5. In the same animals, plasma leakage was also monitored by accumulation of ^{125}I albumin (**B**). In both figures the dotted line represents mean basal accumulation for ease of comparison. Each mediator was injected in duplicate sites per guinea pig. All bars represent the mean ± SD from 2 recipient animals, each animal having received eosinophils from different donor guinea pigs. Histamine was used at $2.5x10^8$ mol/site and ZAP used as 100 µl of a 1/5 dilution.

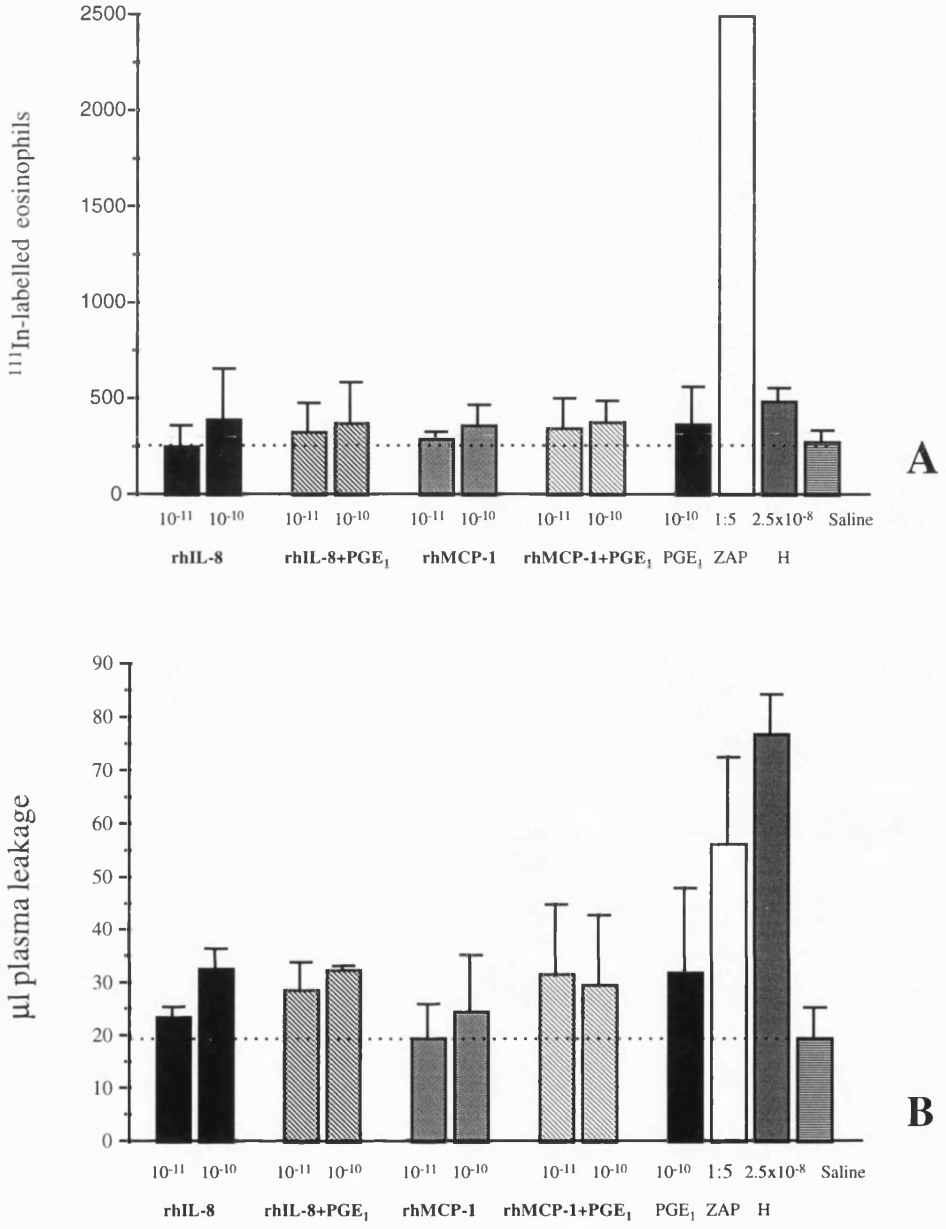


Figure 40 **A**. ^{111}In-labelled eosinophil accumulation in skin sites injected with guinea pig ZAP, but not in response to rhIL-8 and rhMCP-1 +/- PGE_1 (10^{-10} mol/site). In the same animals, plasma leakage was also monitored by accumulation of ^{125}I albumin (**B**). In both figures the dotted line represents mean basal accumulation for ease of comparison. Each mediator was injected in duplicate sites per guinea pig. All bars represent the mean ± SD from 2 recipient animals, each animal having received eosinophils from different donor guinea pigs. Histamine was used at $2.5x10^8$ mol/site and ZAP used as 100 µl of a 1/5 dilution.

5.2.2.1. Migratory responses of blood derived leukocytes to i.d. injections of gpRANTES

Guinea pig naïve skin sites were injected i.d. with gpRANTES or saline and examined histologically for evidence of cellular infiltration. Injection of gpRANTES induced a time and dose-dependent inflammatory response, and the cell counts of extravascular leukocytes are summarised in table 5.3. Within 2 h of gpRANTES injection some evidence of monocytic cuffing around dermal vessels was observed but no overall increase in dermal leukocyte numbers compared to time-matched control samples (figure 41, A-C). A slight neutrophil infiltrate in both the gpRANTES and saline-injected sites at 2 h demonstrated evidence of an acute inflammatory reaction. gpRANTES did not induce eosinophilia whilst sites injected with guinea pig ZAP at this time point were characterised by an eosinophilic and neutrophilic infiltrate (D). At 24 h post injection of 10^{-11} mol/site gpRANTES, an increase in the number of mononuclear cells was observed within the dermis compared to saline-injected sites. At higher doses (10^{-10} mol/site), an increased severity of inflammation was noted with the accumulation of pockets of mononuclear cells in the dermis. At 24 h, only small numbers of eosinophils and neutrophils were present (figure 42). All preparation of histological sections and photography was performed by myself.

Treatment (mol/site)	Time (h)	Sites counted	Leukocyte count
Vehicle	2	4	21±5
gpRANTES 10^{-10}	2	4	30±3
Vehicle	24	6	16±4
gpRANTES 10^{-11}	24	6	40±2
gpRANTES 10^{-10}	24	6	110±10

Table 5.3 The numbers of extravascular leukocytes in the guinea pig dermis at various times following intradermal injection of gpRANTES or saline. Results reflect cell counts from duplicate skin sites in n=3 (24 h) or n=2 (2 h) guinea pigs. Cell counts are expressed as mean ± SEM of cell numbers per hpf (x400). For sites injected with 10^{-10} mol/site gpRANTES (24 h) where pockets of mononuclear infiltrate were observed, counts reflect cell numbers within these areas. No such areas were found in vehicle injected sites.

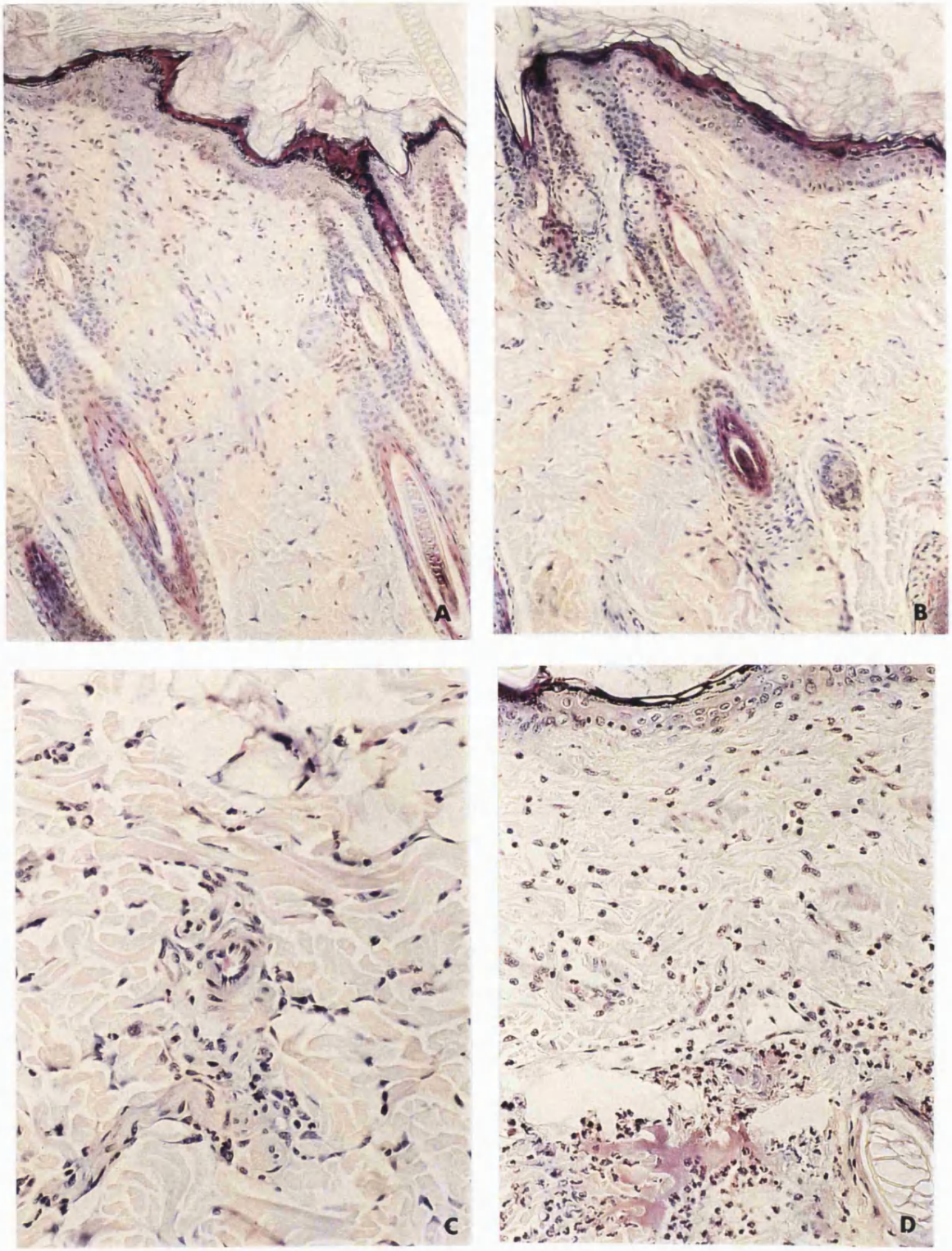


Figure 41. Haematoxylin and eosin-stained representative sections of guinea pig dermal responses to gpRANTES. At 2 h post gpRANTES (10^{-10} mol/site) injection (figure A, x200) no overall increase in dermal infiltrate was observed compared to saline injected sites (figure B, x200), despite some monocytic accumulation around blood vessels (figure C, x400). Only small numbers of eosinophils were observed in gpRANTES and saline-injected sites compared to gpZAP injected sites which was characterised by a granulocytic infiltrate (figure D, x200).

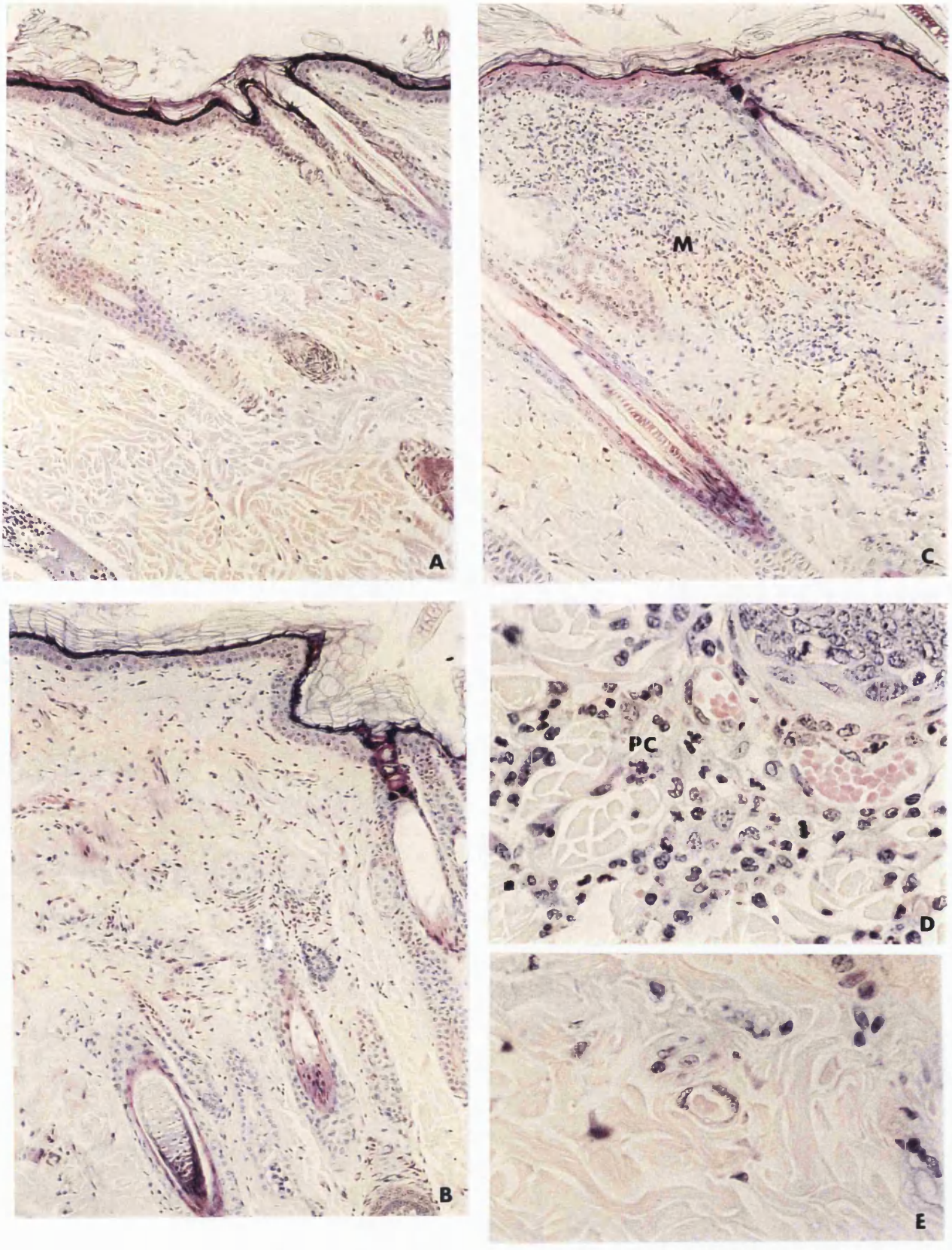


Figure 42. Haematoxylin and eosin-stained representative sections of guinea pig dermal responses to gpRANTES at 24 h. In comparison to saline control sites (figure A, x200), injection of 10^{-11} mol/site gpRANTES caused an increase in the number of monocytes in the dermis (figure B, x200). At higher doses of 10^{-10} mol/site (figure C, x200) large pockets of cells (M) and marked perivascular cuffing (PC) were observed which were predominantly mononuclear (figure D, x1000). Perivascular cuffing was not present in saline controls (figure E, x1000).

5.3. Summary of results:

Characterisation of recombinant gpRANTES protein

- gpRANTES was an effective stimulus of human eosinophils as assessed by increases in $[Ca^{2+}]_i$ in fura-2 loaded cells and chemotaxis responses *in vitro*
- Neither gpRANTES nor hRANTES were able to activate guinea pig peritoneal eosinophils in these assays, even in the presence of IL-5, *in vitro.* Furthermore, *in vivo* studies demonstrated that ^{111}In-labelled guinea pig peritoneal eosinophils did not accumulate in skin sites injected with RANTES.
- However, both guinea pig and hRANTES were potent stimulators of guinea pig peritoneal macrophages.
- gpRANTES exhibited similar potency and efficacy to hRANTES
- A similar profile of activity was observed *in vivo.* Following tracheal instillation of gpRANTES, a dose and time-dependent increase was observed in the number of macrophages but not eosinophils
- In guinea pig skin sites injected with gpRANTES, a monocytic accumulation was observed at 24 h, with no increases in eosinophil numbers above saline-injected sites.

CHAPTER 6. DISCUSSION

The guinea pig has been widely used in the study of allergic airway inflammation. Since the CC chemokine RANTES has been implicated as an important mediator of leukocyte recruitment in allergic asthma (Strieter *et al.* 1996), it is of interest to characterise its role in this species. The work presented in this thesis describes the expression of RANTES in the guinea pig lung as well as the cloning, expression and purification of gpRANTES protein to assess its biological activities.

6.1. The expression of RANTES in the guinea pig lung

RANTES and MCP-1 were expressed in the guinea pig lung and low levels of both chemokines were detected even in non-sensitised/non-challenged animals. Studies by Schall *et al.* (1988) and MacLean (1996a) indicated that such 'constitutive' RANTES expression was also present in the mouse but not in the normal human lung. In the case of the guinea pig, it is possible that chemokine expression in naïve lungs may be indicative of on-going inflammation in these animals.

In a guinea pig model of allergic inflammation, RANTES and MCP-1 appear to be differentially expressed in the lung (figure 5). The use of Northern blot analysis revealed similar levels of message in naive and OA sensitised guinea pig lung following OA challenge. In contrast, MCP-1 mRNA was clearly upregulated at 2, 6 and 12 h compared to time-matched controls. It was not possible to assess chemokine expression at 24 h in this model using Northern blot analysis since degradation of the RNA in these samples appeared to be extensive. Whilst a high susceptibility to RNA degradation appeared to be a feature of guinea pig lung tissue it is possible that the prominent eosinophilia at this time point in sensitised samples might be responsible for excessive

RNase activity. In an attempt to verify chemokine mRNA levels at 24 h post challenge, RT-PCR analysis was performed on a separate group of similarly treated guinea pigs. Using this technique, samples are standardised and therefore comparable by reference to internal levels of a housekeeping gene such as β-actin. As for the Northern blot analysis, no consistent difference was observed in RANTES mRNA levels between naïve and sensitised animals at 2 or 24 h following OA challenge. In comparison, MCP-1 levels increased by almost four fold in the sensitised group compared to controls, at both times (figure 8). The assessment of standardisation levels of chemokines using RT-PCR thus appears to be a suitable alternative to Northern blot analysis.

The kinetic expression of RANTES and MCP-1 in the guinea pig allergic lung has not been previously reported. However, a similar analysis of allergic inflammation in the murine lung by MacLean *et al* (1996) also demonstrated non-variable RANTES expression over 3 to 48 h post OA challenge. In comparison, expression for the CC chemokine eotaxin was strongly induced over a similar time course in OA sensitised but not sham sensitised lungs. Eotaxin is a potent and specific eosinophil attractant originally identified in BAL of OA sensitised guinea pigs at 3-6 h post challenge, by Jose *et al* (1994b). Furthermore this group (Jose *et al.* 1994a) and others (Rothenberg *et al.* 1995b) have demonstrated that OA sensitisation of the guinea pig lung induces increased expression of eotaxin mRNA notably at 3 h following challenge, whilst the chemokine is only weakly expressed in non-sensitised controls.

During clinical asthma however, increased RANTES expression in the lung compared to levels in normal control subjects, implies a more important role in the development of an allergic response, although a number of other CC chemokines are also upregulated.

Bronchial tissue from asthmatics has been reported to express increased levels of RANTES and MCP-3 (Humbert *et al.* 1997) as well as MCP-1 mRNA (Sousa *et al.* 1993). Alam *et al* (1996) demonstrated elevated levels/activity of RANTES, MIP-1α and MCP-1 protein in the BAL of asthmatics, although Teran and colleagues (1996) identified RANTES as the sole eosinophil chemoattractant in BAL fluid and observed a six fold increase in RANTES protein levels at sites of allergen challenge compared to saline challenged sites. In contrast, recent studies by Fahey *et al* (1997) failed to detect any differences in expression of RANTES mRNA between atopic asthmatics and healthy subjects using *in situ* hybridisation. Studies by Kurashima *et al* (1996) have suggested that increased levels of MIP-1α and MCP-1 in the sputum precede and are indicative of the development of a late phase response during acute asthma attacks.

6.2. Potential sources of guinea pig chemokines in the lung

Guinea pig lung tissue was analysed by *in situ* hybridisation to identify cells potentially responsible for the production of RANTES. Initial examination of the sections revealed that overall RANTES mRNA expression was increased in two of three OA sensitised lung sections compared to naïve lungs at 24 h post OA-challenge (figure 11). Analysis of the same samples for eotaxin expression showed no such increases (figure 12). This data appears to conflict with the fairly constant RANTES expression observed using Northern blot analysis. It is particularly surprising since these lungs were also analysed by RT-PCR and overall standardised levels of RANTES or eotaxin were representative of their treatment group. Furthermore other groups (Jose *et al.* 1994b; Rothenberg *et al.* 1995b) have demonstrated variable levels of eotaxin using similar guinea pig models of allergic asthma. Peak expression of eotaxin was detected at 3h following OA challenge

and chemokine expression using *in situ* hybridisation was not analysed at this time point. However, *in situ* hybridisation identifies particular cell types responsible for chemokine expression and studies of expression in the whole lung (even RT-PCT) might not be sufficiently sensitive to detect such subtle changes. Whilst the expression of RANTES appears more strong than for eotaxin, such comparisons based on the intensity of staining are not necessarily accurate. The antisense RANTES riboprobe labelled at twice the efficiency of the antisense eotaxin probe and although a correctional dilution into the hybridisation buffer was made, this also introduces proportionally more unlabelled probe which competes for target mRNA.

In situ hybridisation was successfully used to identify the potential source of RANTES and for comparison, eotaxin. Both chemokines portrayed a similar pattern of expression, predominantly within the alveolar macrophage and less so in the endothelium, bronchial epithelium and cells lining the alveoli.

6.2.1. RANTES and eotaxin expression in the alveolar macrophage

In situ analysis indicated that the alveolar macrophage expressed RANTES and eotaxin mRNA in guinea pig lung tissue. Although the number of BAL macrophages was increased in the animals which were sensitised to OA (A.-M. White, personal communication) increased numbers of macrophages within the lung section were not observed. The identification of guinea pig mononuclear cells as a potential source of RANTES was supported by Northern blot analysis of total RNA extracted from peritoneal macrophages cultured *in vitro* (figure 17). No constitutive expression was observed in these cells but was prominent following LPS-stimulation at all time points analysed. Since LPS has been shown to induce rapidly TNFα mRNA within 1 h in

rodent alveolar macrophages (Ulich *et al.* 1991), it is possible that at least some of the observed RANTES expression in guinea pig peritoneal macrophages was TNFα mediated. Furthermore, work in this thesis indicates that recombinant gpRANTES itself induces RANTES expression in these cells and although it is not clear whether the expression is associated with production of the protein, it does raise the possibility of a RANTES-mediated positive feedback mechanism. Further work is needed to investigate the expression of RANTES and eotaxin in the guinea pig alveolar macrophage especially since Van Otteren *et al* (1994) demonstrated that MIP-1α appears to be secreted at far greater levels from LPS-stimulated murine alveolar macrophages compared to those isolated from the peritoneum.

A number of other workers have identified the macrophage as a source of RANTES. Schall *et al* (1992) initially isolated mRANTES from the macrophage cell line PU5-1.8. Devergne *et al* (1994) reported RANTES in alveolar macrophages (and epithelial cells) in delayed-type hypersensitivity granulomas and confirmed this finding by observing RANTES expression and protein release in IFNγ-treated BAL macrophages. In contrast, Van Otteren *et al* (1995b) implicate the endothelial cell but not the alveolar macrophage as a source of RANTES in rodent models of endotoxaemia. Such variations could reflect the nature of the stimulus, for example TNFα has been shown to be a key mediator of sepsis and is also the most potent inducer of RANTES expression in a number of cells including the epithelium. In contrast the upregulation of IFNγ, IL-1β and TNFα is a specific feature of DTH granulomas and might favour stimulation of macrophages and endothelial cells (Devergne *et al.* 1994). Interestingly, a recent study by McKee and colleagues (1996) reported that alveolar macrophages from patients with idiopathic pulmonary fibrosis express RANTES as well as MIP-1α and β and MCP-1

mRNA following stimulation with fragments of the glycosaminoglycan, hyaluronan. Such fragments are generated from the high molecular weight parent polymer, which did not induce expression, during chronic inflammation although it is not clear whether such fragments are present in allergic lung inflammation.

6.2.2. RANTES and eotaxin were not expressed in other leukocytes of the lung

Alveolar macrophages were the only leukocytes identified in the guinea pig lung to express RANTES or eotaxin (figures 11 and 12). Schall *et al* (1990) originally identified RANTES expression in IL-2 dependent human T cell lines and studies by Conlon and colleagues (1995) showed that a number of human peripheral blood lymphocytes especially CD8+ CD45RO+ T cell, secreted RANTES as well as MIP-1α. It is feasible that the scant numbers of these cells in the lung made analysis of their chemokine expression difficult. In this study, all eosinophils identified in the guinea pig lung did not express RANTES or eotaxin. In contrast, a recent study by Ying and colleagues (1996) concluded that human eosinophils synthesis RANTES through *de novo* gene transcription, identifying over 50% of the immunopositive cells for RANTES during allergen-induced late-phase cutaneous reaction as $EG2^+$ eosinophils. The authors confirmed the human eosinophil to be a source of RANTES by demonstrating the release of biologically active protein from human peripheral blood eosinophils after stimulation with serum-coated particles, findings supported by Lim *et al* (1996) using GM-CSF stimulated cells. Recent work by Garcia-Zepeda (1996) suggests that the human eosinophil might also be a source of eotaxin.

6.2.3. The epithelium and endothelium express RANTES and eotaxin mRNA

Our *in situ* hybridisation studies suggest evidence of RANTES and eotaxin expression in airway epithelium and less so in the endothelium (figures 11 and 12). These proteins join an ever expanding list of other chemokines produced by these cells, including IL-8 (Standiford *et al.* 1990a; Hebert *et al.* 1990) and MCP-1 (Sousa *et al.* 1994). In terms of leukocyte migration, these cells could be thought of as 'gate keepers', dictating which cells pass into the bronchial lumen and extravascular spaces through the generation of high, localised concentrations of chemokines. It seems feasible that RANTES and eotaxin production at these sites contributes towards leukocyte accumulation in the BAL and lung of the guinea pig and characterisation of the biological activity of these chemokines suggests that RANTES mediates macrophage accumulation, whilst eotaxin, eosinophil accumulation (this work and Jose *et al.* 1994b). Since both endothelial and epithelial cells seemingly secrete a similar array of chemokines, the balance of leukocytes in the lung tissue itself (compared to the bronchial lumen) might be mediated by the differential expression of adhesion molecules. Studies by Weg *et al* (1993) demonstrated that an antibody to VLA-4 inhibited eosinophil accumulation in the guinea pig skin following a passive cutaneous anaphylaxis reaction, implying that VLA-4-mediated adhesion might be important for transendothelial migration. Milne and Piper (1993) suggested that CD18/ICAM-1 interactions control migration across the epithelium into the bronchial lumen, since pretreatment of OA sensitised guinea pigs with anti-CD18 mAbs decreased the number of eosinophils in the BAL but not in the lung tissue. Das and colleagues (1995) also reported that the administration of antibodies to both VLA-4 and CD18 were required to inhibit BAL eosinophilia in the guinea pig but suggested that both eosinophils and monocytes could use both mechanisms to cross either barrier. However, it is clear that chemokines themselves can

influence adhesion molecule expression; RANTES upregulates CD11b/CD18 on eosinophils (Alam *et al.* 1993) and appears to induce eosinophil transendothelial migration through HUVEC via CD18 and VLA-4-dependent mechanisms (Ebisawa *et al.* 1994). However, recent work by Burke-Gaffney (1996) suggests that eotaxin, but not RANTES or MIP-1α, mediates VLA-4-dependent eosinophil adhesion to human lung microvascular endothelial cells.

Our observations of RANTES and eotaxin production from endothelial and epithelial cells supports those of a number of other workers. Marfaing-Koka *et al* (1995) reported RANTES expression in human endothelial cells following synergistic induction with TNFα and IFNγ which could be inhibited with IL-4 and IL-13. Rothenburg and colleagues (1995a) similarly described eotaxin expression in a murine endothelial cell line, although interestingly, this group also showed that eotaxin production was associated with IL-4 production from transfected tumours *in vivo*. The expression of RANTES in cultured epithelium similarly seems to require cytokine stimulation. Kwon *et al* (1995) observed RANTES but not MIP-1α expression in the epithelial cell line A549 upon induction by TNFα or IL-1 (with peak expression at 24 and 48 h respectively) with RANTES immunoreactivity increasing for both stimuli at 48 h. Wang and colleagues (1996) observed TNFα-induced RANTES release from primary bronchial epithelial cells peaking slightly earlier at 24 h. This group also demonstrated epithelial RANTES protein expression in bronchial biopsy sections from mild asthmatics although *in situ* hybridisation studies by Humbert *et al* (1997) fail to support their findings. Many of these investigators have demonstrated reduced RANTES expression *in vitro* and *in vivo* following glucocorticoid treatment (Kwon *et al.* 1995;

Wang *et al.* 1996; Stellato *et al.* 1995) It has been suggested that a down regulation of transcription factors, notably NF-κB, could be responsible for these effects (Wang *et al.* 1996). The 5'-flanking region of the human RANTES gene contains an array of transcription factor binding sites and the rapid induction of RANTES following IL-1β stimulation in these cells is concurrent with increased binding activity of NF-κB (Danoff *et al.* 1994).

The *in situ* analysis also revealed weak positive staining for RANTES and eotaxin mRNA within the alveolar wall (figures 11 and 12). Increased expression, particularly for eotaxin, may have been observed at 3 h in sensitised tissues, although this time-point was not investigated. This layer consists primarily of an epithelium of type I and II pneumocytes (although type I cells are infrequently visible in histological section by virtue of their extremely flattened morphology), endothelial cells, connective tissue including fibroblasts as well as the readily identifiable macrophage, embedded within the wall. In absence of double staining procedures, the precise stromal/parenchymal cells expressing these chemokines are not readily identified *in situ*, although Van Otteren *at al* (1995b) reported TNF-induced RANTES protein release from rat type II pneumocytes and Lukacs and colleagues observed RANTES expression predominantly in these cells during allergic inflammation in the mouse lung (Lukacs *et al.* 1995b).

6.2.4. Expression of chemokines in guinea pig lung fibroblasts

The fibroblast has been reported as a source of a number of chemokines and although these cells are not readily identified *in situ*, they are proliferative in culture. Using Northern blot anaysis, no detectable expression of RANTES or eotaxin was observed in a guinea pig fibroblast cell line, JH4-Cl1, or fibroblast-like primary cells grown from

lung explants. This suggests that the lung fibroblast does not play a key role in the production of these chemokines (figures 18 and 19). In contrast, RANTES production has been reported from cytokine-stimulated human dermal fibroblasts as a truncated form, $[Tyr\text{-}RANTES]_{68}$ (Noso *et al.* 1995). Furthermore, rheumatoid synovial fibroblasts expressed RANTES mRNA following TNFα or IL-1β stimulation (Rathanaswami *et al.* 1993). No chemokine expression was observed in guinea pig fibroblasts following stimulation with these recombinant human cytokines, presumably a consequence of differences in species specificity. Given that the human macrophage is a rich source of IL-1 and TNFα (Ulich *et al.* 1991), supernatant from LPS-stimulated guinea pig peritoneal macrophages was assumed to contain guinea pig TNF and IL-1 and small amounts of IFNγ, although the exact levels of these were not determined. However Rolfe *et al* (1992) demonstrated that chemokine expression in human pulmonary fibroblasts stimulated with LPS-treated alveolar macrophages could be obliterated using anti TNF and anti IL-1β antibodies. Rathanaswami *et al.* (1993) demonstrated that RANTES expression in synovial fibroblasts was inhibited by dual TNFα and IL-1β stimulation but augmented by co-stimulation with IFNγ. It seems unlikely that the possible presence of both TNFα and IL-1 would have diminished any RANTES message in the guinea pig fibroblasts, since stimulation with recombinant guinea pig TNFα in a later experiment also did not induce expression. Furthermore, even in the presence of conditioned media from Con A-stimulated spleen cells, that was assumed to contain guinea pig IFNγ (Trinchieri & Perussia, 1985), no RANTES expression was induced although it is possible that IFNγ was not present at optimal doses.

The guinea pig lung fibroblast appears to be important in the production of other chemokines such as MCP-1 and IL-8, and their expression at early time points is consistent with descriptions of these chemokines being rapidly induced following stimulation (Miller & Krangel, 1992). It is not clear why a high expression of MCP-1 was seen at early time points in vehicle-treated primary fibroblasts which became stimulus dependent after 12 h (figure 19). This might reflect a time dependent response to the addition of fresh medium containing FCS. However the 'early' expression of MCP-1 in the guinea pig fibroblast corresponds with that observed in the sensitised whole guinea lung notably at 2 h post OA challenge and it is possible that this cell is a key contributor of the overall expression of MCP-1. A similar time-course for MCP-1 expression was reported by Rolfe and colleagues (1992) in human pulmonary fibroblasts following TNFα or IL-1β stimulation. This group also demonstrated MCP-1 expression after stimulation with conditioned media from LPS-treated human alveolar macrophages and suggested that a positive feedback loop between monocyte and fibroblast involving MCP-1, might maintain infiltration of monocytic cells into the lung. gpMCP-1 is able to stimulate guinea pig peritoneal macrophages (Yoshimura 1993), and it is possible such a system exist in the guinea pig.

6.3. The characterisation of gpRANTES protein

6.3.1. Sequence homology of gpRANTES

gpRANTES is highly homologous with hRANTES at both the nucleotide (90%) and amino acid (91%) level, as well as with murine RANTES (89% and 88% respectively) over the region of the secreted protein (figure 20). In comparison to other guinea pig CC chemokines cloned to date, gpRANTES shares 28% amino acid homology with

eotaxin, 37% with gpMCP-1 and these latter proteins are considerably less well conserved with their human counterparts (62% and 56% respectively, Ponath (1996), Yoshimura (1993). Furthermore, the high homology of gpRANTES with mRANTES is quite distinct from other chemokines. Eotaxin and gpMCP-1 are only 63% and 45% identical with their murine counterparts (Rothenberg *et al.* 1995a; Yoshimura, 1993) whilst IL-8 has not been identified in mouse or rat (Yoshimura & Johnson, 1993). Yoshimura (1993) suggested that these observations might support the theory that the guinea pig has evolved from a separate lineage from other rodents (Graur *et al.* 1991).

6.3.2. Expression of gpRANTES by mutation of hRANTES cDNA

High levels of expression in *E. coli* were obtained by mutating the hRANTES cDNA at 6 positions to achieve the predicted amino acid sequence for gpRANTES (figure 25), although expression systems utilising gpRANTES cDNA were unsuccessful (figure 21). It is clear that the N terminus is important in conferring activity for human CC chemokines. Proudfoot and colleagues (1996) demonstrated that retention of methionine at the N terminus of the mature hRANTES protein, a phenomenon common to *E. coli* derived proteins, renders the protein inactive as an agonist but effective at inhibiting RANTES and MIP-1α-induced responses in THP-1 cells. Methionine retention by gpRANTES was successfully prevented by expressing the protein with a cleavable N terminal hexapeptide leader sequence ending in Arg at the 5' end, as previously described (Proudfoot *et al.* 1995). Amino terminal sequencing showed that the initiating methionine was removed from the mature gpRANTES and this protein induced changes in $[Ca^{2+}]_i$ in the pro-monocytic cell line THP-1 (figure 25B), which has been reported to be RANTES-responsive by virtue of CCR1 receptor expression (Wang

et al. 1993; Proudfoot *et al.* 1995). In comparison, hexapeptide-gpRANTES failed to induce any response in these cells.

A number of groups have also demonstrated that N terminal deletions of the mature chemokine itself can vastly alter activity of the protein. Studies by Clarke-Lewis and co-workers (Gong *et al.* 1996) indicated that N terminal truncations to hRANTES (9-68) as well as MCP-1 (9-76) and MCP-3 (10-76) abolished agonist activity but conferred inhibitory action towards monocytic responses induced by all three parent chemokines. Interestingly, a single amino acid deletion from the N terminal residue of MCP-1, changed the target cell selectivity from a basophil to an eosinophil activator (Weber *et al.* 1996). However, Noso and colleagues (1995) recently reported that an N terminally truncated form of hRANTES was the major eosinophil attractant (as well as GMCSF) released from dermal fibroblasts which showed identical potency and efficacy in a chemotaxis assay system to the natural $[Ser\text{-}RANTES]_{68}$.

Clearly the identification of a native source of gpRANTES protein would allow confirmation of the N terminus (i.e. the position at which removal of the presumed signal sequence takes place). Human platelets harbour stable mRNA species for a number of CXC chemokines in addition to MCP-3 and RANTES (Power *et al.* 1995) and are a rich source of RANTES protein (Kameyoshi *et al.* 1992) which appears to be localised, along with MIP-1α, within the α granules (Klinger *et al.* 1994). Extensive attempts for this project to detect RANTES in guinea pig platelets (figures 26 and 27) and independently by J.-M. Schröder (University of Kiel, personal communication), who analysed platelet HPLC fractions for human eosinophil chemoattractant activity, have proved unfruitful. Furthermore, although the guinea pig macrophage expresses RANTES mRNA, it was not clear whether this is utilised for protein synthesis.

Therefore, it had to be assumed that gpRANTES has the same N terminus as hRANTES.

6.3.3. gpRANTES activates monocytic cells but not eosinophils in the guinea pig

RANTES demonstrates selectivity between guinea pig peritoneal eosinophils and macrophages. gpRANTES and hRANTES were not able to cause migration (figure 30) or an increase in $[Ca^{2+}]_i$ (figure 33) in guinea pig peritoneal eosinophils. ^{111}Indium-labelled guiena pig peritoneal cells did not accumulate in skin sites in donor guinea pigs injected with human RANTES (figure 38). Furthermore, it would appear that these cells do not migrate towards RANTES even when co-incubated with IL-5 (figure 32). In contrast, IL-5 has previously been demonstrated to enhance the chemotactic response of human eosinophils to different chemokines, including RANTES (Ebisawa *et al.* 1994; Schweizer *et al.* 1994). However, both human and gpRANTES were potent activators of human blood eosinophils. It was not possible to assess the RANTES responsiveness of blood derived guinea pig eosinophils (or indeed monocytes) directly *in vitro* mainly due to the low circulating numbers of these cells in normal animals. Thus, attempts were made to boost the eosinophil count by i.v. injection of IL-5, which induces eosinophil release from the bone marrow according to a method by Collins *et al.* (1995). However, analysis of blood smears and leukocyte preparation cytospins from these animals showed that many cells were not well differentiated. Separation of the leukocyte subtypes proved difficult using a Percoll gradient and furthermore, guinea pig neutrophils did not recognise the human CD16 mAb bound to MACS beads, which allowed effective separation of human peripheral blood eosinophils through negative selection. Examination of the chemotactic response of the blood leukocytes from these animals revealed a similar migratory response towards RANTES as for the control.

Macrophages from the guinea pig peritoneum were responsive to RANTES as demonstrated by changes in $[Ca^{2+}]_i$ and chemotaxis (figure 34). RANTES induces an immediate and transient rise in $[Ca^{2+}]_i$, and the chemotaxis studies were performed over a relatively short (2-4 hours) incubation period. Therefore, it seems reasonable to assume that RANTES acts directly on these cells, rather than inducing an intermediate effector. The profile of RANTES activity in the guinea pig is clearly different from human peripheral blood cell populations, where RANTES is a potent activator of both eosinophils and monocytes (this work and references by Schall *et al.* (1990) and Rot *et al.* (1992). This difference does not appear to be accounted for by species variation between the primary sequence of guinea pig and human RANTES since, where tested, these proteins have almost identical biological activity.

It could be argued that differences between the human and guinea pig eosinophil populations used in these *in vitro* experiments reflect the use of blood-derived cells compared to cells isolated from an inflammatory site. Receptor desensitisation may occur following extravasation. However, guinea pig peritoneal eosinophils are able to respond to other chemoattractants such as C5a and LTB_4 (Faccioli *et al.* 1991) and when primed with IL-5, to IL-8. Moreover, Jose *et al* (1994b) have demonstrated responsiveness of elicited guinea eosinophils towards eotaxin. Furthermore, it is clear that the use of elicited cells *per se* does not preclude stimulation by RANTES, as it is an effective activator of peritoneal macrophages.

The *in vivo* analysis indicates that, as *in vitro,* gpRANTES has a selective action as a monocyte/macrophage attractant. Following instillation of gpRANTES into the guinea pig airways, a significant increase in BAL macrophage numbers, but not eosinophils, was observed. This effect was dose-dependent and the mononuclear cell accumulation

was detectable at 6 hours and sustained for at least 48 hours (figure 37). Similarly, injection of gpRANTES into naïve guinea pig skin sites, induced a monocytic infiltration which was dose and time dependent. No evidence of an increase in eosinophil numbers compared to saline-injected sites was observed at 2 or 24 hours post injection, whilst large areas of dermal macrophages were observed at 24 hours (figures 41, 42). Intradermal injections have been shown to be a valid model of blood-derived eosinophil migratory responsiveness towards RANTES by Meurer *et al* (1993) who reported eosinophilic and monocytic infiltration in skin sites of the dog in response to hRANTES at 4 h post injection which was vastly elevated at 24 h. Interestingly, the authors observed a six fold increase in dermal macrophage numbers using a dose of 500 pmol/site hRANTES compared to saline sites. Results from this thesis show that RANTES injection into guinea pig skin sites induces a similar fold increase using only 100 pmol/site although no larger doses were injected to assess whether the response was maximal at 100 pmol/site. RANTES has been shown to be an important mediator of macrophage recruitment *in vivo* in a murine model of endotoxemia. Passive immunization with anti-RANTES antibodies resulted in a significant reduction in macrophage infiltration into the lung at 24 hours following intraperitoneal administration of lipopolysaccharide (VanOtteren *et al.* 1995a).

A number of recent reports have shed light on factors which mediate eosinophil accumulation in the guinea pig *in vivo*. The CC chemokine eotaxin induces eosinophil activation *in vitro* (Jose *et al.* 1994b) and Collins *et al* (1995) demonstrated eosinophil accumulation in guinea pig skin sites injected with eotaxin. This accumulation was increased six fold in animals preinjected i.v. with IL-5. The authors proposed a systemic effect of IL-5 to mobilise large numbers of bone marrow eosinophils into the blood which would subsequently be recruited by high concentrations of local

chemokine. Eosinophil migration might also be facilitated by the effects of IL-5 priming (Schweizer *et al.* 1994). However, it seems unlikely that the use of IL-5 would unveil such a role for RANTES *in vivo* in the guinea pig under similar circumstances, since RANTES-induced migration of guinea pig IL-5-primed eosinophils was not observed *in vitro*, and eotaxin itself is a potent eosinophil attractant (Jose *et al.* 1994b). IL-5 is associated with a Th2 response and pretreatment of OA sensitized guinea pigs with anti-IL-5 antibody TRFK-5, prior to challenge reduces airway hyperreactivity and the associated eosinophilia (Van Oosterhout *et al.* 1993). In the mouse, a number of groups have reported that IL-4 rather than IL-5 is causally related to the eosinophilia of allergic inflammation (Lukacs *et al.* 1994) and disruption of the murine IL-4 gene blocks Th2 cytokine responses (Kopf *et al.* 1993).

In addition to these cytokines, murine allergic responses are associated with TNFα (Lukacs *et al.* 1995a) which probably acts through a combination of adhesion molecule upregulation, initiation of cytokine cascades and induction of key chemokines such as eotaxin and MIP-1α (Lukacs *et al.* 1996a). Most recently White *et al* (1996) have demonstrated gpTNFα-induced eosinophilia in the guinea pig BAL, although the precise chemoattractants have yet to be elucidated since gpTNFα itself has no direct eosinophil attractant ability.

6.3.4. Evidence for chemokine receptors on guinea pig peritoneal cells

Studies as part of this project indicate that gpRANTES activates guinea pig peritoneal macrophages with similar potency to hRANTES, and hMIP-1α and hMIP-1β are also agonists for these cells (figure 35). However, possible receptors that bind RANTES on guinea pig monocytic cells have yet to be identified, although Van Riper *et al* have

shown some specific binding of hRANTES to guinea pig mononuclear cells (1993). All five human CC chemokine receptors cloned to date are expressed on monocytic cells and of these all but the MCP receptor, CCR2, bind RANTES (reviewed by Power and Wells 1996) although the reason for such biological redundancy is not clear. Of particular note is the CCR5 which binds MIP-1α and β as well as RANTES and may be important in the selective recruitment of monocytic cells since it is expressed on primary adherent monocytes but not eosinophils or neutrophils (Combadiere *et al.* 1996). It is also noteworthy that of the four murine CC receptors which have been cloned (homologues of human receptors CCR1-4) all preferentially bind MIP-1α and most also bind RANTES (Post *et al.* 1995; Meyer *et al.* 1996; Hoogewerf *et al.* 1996).

Marleau and colleagues (1996) demonstrated that hRANTES and hMCP-3 were able to bind receptors on guinea pig peritoneal eosinophils, displacing eotaxin. However, unlike eotaxin they were unable to induce changes in $[Ca^{2+}]_i$ in these cells. MIP-1α/β did not bind or activate these eosinophils. Furthermore hRANTES inhibited the accumulation of ^{111}In-labelled eosinophils in eotaxin-injected guinea pig skin sites. This implies that a common receptor on these cells binds RANTES, eotaxin and MCP-3, and has lead to the suggestion that hRANTES could antagonise and regulate eotaxin responses in the guinea pig. gpRANTES is also inactive towards these cells (this thesis), although its ability to displace eotaxin and thus inhibit eotaxin-induced responses remains to be established. However, should such a role exist, then the co-localised production of eotaxin with RANTES in the guinea pig lung as demonstrated by *in situ* hybridisation mRNA distribution, might facilitate such inhibition. On the bases of binding data it would appear that this receptor is the guinea pig homologue of the human eotaxin receptor, CCR3 which also binds eotaxin, MCP-3 and RANTES with

K_d of 0.1, 2.7 and 3.1 nanomolar respectively (Daugherty *et al.* 1996). This receptor is expressed at high levels on the human eosinophil. However since RANTES as well as MCP-3 and eotaxin are able to induced changes in $[Ca^{2+}]_i$ in AML.3D10 cells transfected with the receptor, RANTES is presumably able to induce human eosinophil responses by signalling through this receptor. Human eosinophils also express the CCR1 receptor (Proudfoot *et al.* 1995) which binds RANTES at a higher affinity than CCR3 (Kd of 0.6 nM) but is present at much lower levels (Daugherty *et al.* 1996). It is not known whether guinea pig eosinophils express CCR1. However since neither RANTES, hMIP-1α or MCP-3 (own observations and Marleau *et al.* 1996), the ligands for human CCR1, activate guinea pig eosinophils it is tempting to speculate that this receptor is absent on these cells. A summary of postulated guinea pig and known human receptors for eosinophils and macrophages are summarised in diagram 6.1.

The inability of RANTES to induce eosinophil activation is not confined to the guinea pig; eosinophils from IL-5 transgenic mice are also unresponsive to RANTES (Post *et al.* 1995). However, homologues of CCR1 and CCR3 have been identified on murine eosinophils (Post *et al.* 1995; Gao & Murphy, 1995; Meyer *et al.* 1996) which bind RANTES as well as MIP-1α and MIP-1β, and MIP-1α is a potent activator of these cells (Lukacs *et al.* 1996c). It is possible that the CC chemokines (RANTES, MCP-3, eotaxin and MIP1-α) bind homologues of the same eosinophil receptors with differing affinities or efficacies according to the species. Such a rationale would explain the species differences in CC chemokine involvement in allergic inflammation, since the expression of an eosinophil attractant appears to correlate with induction of pulmonary eosinophilia. In a model of murine airway inflammation induced by challenge with Schistosoma mansoni egg antigen, Lukacs *et al* (1996c) demonstrated increases in MIP-

1α expression at 8 h post-challenge. Pretreatment with anti-MIP-1α neutralising antibody prior to challenge decreased BAL eosinophil numbers by 50 %, consistent with the role of MIP-1α as a murine eosinophil attractant. In the human, a number of eosinophil-attracting chemokines are upregulated during allergic responses in the lung, including RANTES (Teran *et al.* 1996).

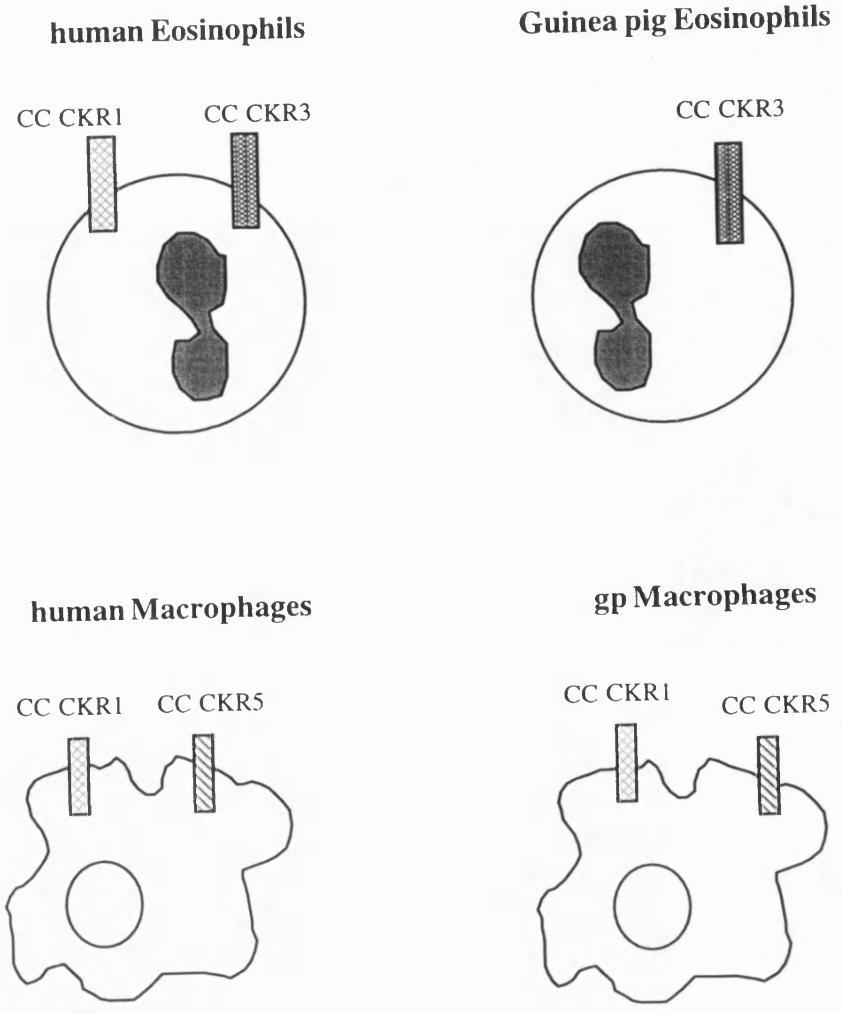


Diagram 6.1. Postulated guinea pig and known human CC chemokine receptors on eosinophils and macrophages.

Work presented in this thesis demonstrates that the expression of RANTES mRNA was not induced in response to antigen sensitisation/challenge in the guinea pig and is not an eosinophil attractant in this species. gpRANTES is however a potent activator of guinea pig monocytic cells and since macrophages are the predominant cell type in BAL in the absence of inflammation and a low level expression of RANTES mRNA was detected in normal guinea pig lung, it is tempting to speculate that RANTES may contribute to the maintenance of resident lung macrophages. Other authors have shown that gpMCP-1 activates guinea pig elicted, but not resident macrophages, and eotaxin is a potent guinea pig eosinophil-attractant and may be particularly effective in conjunction with Th2-type cytokine IL-5. Since these two chemokines are both upregulated in the OA-challenged lung they may be more important than RANTES in cell recruitment during allergic inflammation in the guinea pig.

A central role for chemokines in asthma remains to be established and the determination of the action of any one chemokine in allergic airway inflammation can only be made in an appropriate animal model. Although these results indicate that RANTES has differing cellular selectivity in the guinea pig compared to the human, the guinea pig may still be suitable for the assessment of potential therapeutic reagents at the level of the chemokine receptor.

6.4. Further work

This thesis has demonstrated evidence for RANTES as a macrophage attractant in the guinea pig. This would appear to be relevant to monocyte recruitment in the lung since tracheal instillation of gpRANTES increases BAL macrophage numbers. However, the *in vitro* characterisation of gpRANTES centred around the responses of peritoneal cells. It may be more relevant to assess the RANTES responsiveness of guinea pig alveolar macrophages, as well as CC chemokine expression in these cells.

Recent work by Marleau and colleagues (1996) suggests the presence of a shared eotaxin/RANTES/MCP-3 receptor on guinea pig peritoneal eosinophils. Cloning of such a receptor would allow a full characterisation of chemokine responses and cast light on reasons for the ability of RANTES and MCP-3 to bind but not activate guinea pig eosinophils. As for other chemokines, key sequences for receptor binding can be elucidated by the synthesis and biological characterisation of truncated forms of the protein and in this way receptor antagonists designed. A feature of the chemokines is the multiplicity of proteins binding to any one receptor although the reasons for this redundancy are unclear. A number of authors have therefore questioned the likelihood that inhibition of an individual chemokine would adequately affect the overall inflammatory process, leading to suggestions that antagonism at the level of the receptor is a more prudent target for therapeutic intervention (Teixeira *et al.* 1995). Alternatively, the inhibition of central cytokines, particular IL-5, IL-4 and TNFα, might inhibit production of a number of down-stream chemokines.

Work in this thesis indicates that RANTES might not be central to eosinophil recruitment in allergic inflammation in the guinea pig. However, studies of RANTES as a histamine releasing factor from human basophils (Kuna *et al.* 1993) and mast cells

(Mattoli *et al.* 1995) suggest that RANTES might indirectly induce bronchoconstriction and other features of the immediate phase of an allergic response. Studies are currently underway to determine whether such a role exists for RANTES in the guinea pig (M.Watson, personal communication).

It remains to be seen what relative contribution the chemokines make to the overall development and maintenance of allergic inflammation. How, for example, might they interact with the non-specific leukocyte attractants PAF and the leukotrienes, equally implicated in the pathogenesis of asthma by virtue of their upregulation? Studies that embrace the many inflammatory molecules deciphered over the last few decades will lead to a greater understanding of mechanisms which ultimately result in the selective recruitment of leukocytes.

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Appendix 1: Media and Solutions

Where appropriate, media and solutions were sterilised by autoclaving for 20 min at 15 lbs/sq inch (121 °C) on a liquid cycle. All concentrations are final.

DNA loading buffer: 0.5 % SDS, 25 % glycerol, 0.25 % bromophenol blue, 0.05 M EDTA

Hybridisation solution for cDNA probes: High SDS buffer contains 7 % SDS, 50 mM sodium phosphate buffer pH 7.0, 2 % blocking reagent, 5x SSC, 0.1 % Sarcosyl, 50 % formamide. Warm to 50°C to dissolve.

Hybridisation solution for riboprobes: 50 % deionised formamide with 5x SSC warmed to 50 °C before addition of 10 % dextran sulphate. After complete solubilisation at 50 °C the following reagents were added; 5x Denhardt's solution, 0.5 % SDS, 1 % denatured sheared herring sperm DNA and DEPC-treated water to required volume.

Miniprep solution I: 25 mM Tris containing 50 mM glucose and 10 mM EDTA

Miniprep solution II: 0.2 N NaOH containing 1 % SDS prepared from sterile stocks of 10 N NaOH and 20 % SDS on day of experiment.

Miniprep solution III: 3 M potassium acetate containing 11.5 ml glacial acetic acid

MOPS buffer (x20): 0.8 M MOPS, 0.02 M EDTA, 0.2 M sodium acetate, adjusted to pH 7.0 with NaOH pellets.

NBT/BCIP alkaline phosphatase substrate: 0.03 % NBT and 0.015 % BCIP in 100 mM Tris HCl adjusted to pH 9.5, containing 5 mM $MgCl_2$ and 100 mM NaCl.

Northern blot maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl adjusted to pH 7.5 with NaOH pellets, DEPC-treated overnight then autoclaved.

Northern blot blocking buffer stock: 10% blocking reagent for nucleic acids in maleic acid buffer. Microwaved until boiling to dissolve.

Northern blot chemiluminescence substrate buffer: 0.1 M Tris, 0.1 M NaCl, 50mM $MgCl_2$. Adjusted to pH 9.5.

Paraformaldehyde solution (4 %): 4 % paraformaldehyde, 2 % sucrose, 5 mM $MgCl_2$, 0.02 % DEPC in 1x PBS, pH 7.4.

RNA loading buffer: 33 % glycerol in DEPC-treated water with 0.25 % bromophenol blue

RNA sample buffer: 8 % formaldehyde (from stock of 37 %), 5x MOPS, 0.6 % w/v ethidium bromide, 60 % v/v formamide.

LB medium (Luria-Bertani medium): 1 % w/v Bacto tryptone, 0.5 % Bacto yeast extract, 1 % NaCl, pH 7.0 with NaOH. LB plates were made from 2 % agar in LB medium.

Recovery medium: 2 % w/v Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

Sodium acetate solution (3 M): 40.8 % sodium acetate solution (in DEPC-treated water) was adjusted to pH 5.2, by addition of an approximately equal volume of glacial acetic acid.

SSC (x20): 3 M NaCl, 0.3 M trisodium citrate, pH 7.0. DEPC-treated and autoclaved.

TBE (x5): 54 g/L Tris base, 27.5 g/L boric acid, 4.65 g/L $Na_2EDTA.H_2O$, pH 8.3

TBS: 0.9 % w/v NaCl in 10 mM Tris HCl pH 7.4

Tris-saturated phenol/chloroform commercial preparation containing: Tris (0.1 M) - saturated phenol:chloroform:isoamyl alcohol, 25:24:1

Western blocking buffer: 5 % non-fat powdered milk (Marvel), 0.05 % azide in 1x TBS

Western electrophoresis running buffer: 25 mM Tris containing 192 mM glycine and 0.1 % SDS

Western protein sample buffer x5: 50 % glycerol, 10 % SDS, 1.5 % 2-mercaptoethanol and 0.5 mg bromophenol blue in ¼ dilution of the stacking gel buffer

Western transfer buffer: 25 mM tris containing 192 mM glycine and 20 % methanol

Western gel components:

Component	*Running gel (15 %)*	*Stacking gel (4 %)*
Distilled water	1.175 ml	2.15 ml
10 % SDS (w/v)	50 µl	37.5 µl
1.5 M Tris buffer pH 8.8 (Running gel buffer)	1.25 ml	-
0.5 M Tris buffer pH 6.8 (Separation gel buffer)	-	1 ml
Protogel*	2.5 ml	0.63 ml
TEMED	5 µl	5 µl
10 % Ammonium persulphate	50 µl	37.5 µl

* Protogel (national Diagnostics, Atlanta, USA) consists of 30 % w/v acrylamide and 0.8 % bisacrylamide

Appendix 2: Components of commercial reagents

Denhardt's solution: contains BSA, Ficoll, PVP in water, as a 50x concentrate

Diff Quik: Solution I contains Eosin G in phosphate buffer pH 6.6 (1.22 g/L)

Solution I contains thiazine dye in phosphate buffer pH 6.6 (1.1 g/L)

Fixative contains fast green in methanol (0.002 g/L)

Euthatal: sodium pentobarbitone (60 mg/ml)

Hypnorm: fentanyl citrate (0.315 mg/ml) with fluanisone (10 mg/ml)

^{111}Indium Chloride ($^{111}InCl_3$): 10 μCi/ml in pyrogen-free 0.04 N HCl

^{125}Iodine labelled human serum albumin (^{125}I-HSA): 20 mg albumin/ml of sterile isotonic saline 50 μCi/ml

Lambda DNA-EcoR I and Hind III digests for molecular weight markers

Double stranded DNA was digested with EcoR I and Hind III restriction endonucleases and dialysed against 10mM Tris-HCL pH 7.8 containing 1 mM EDTA. The digest consisted of 13 fragments from 125 base pairs to 21,226 bases.

Mark 12, wide-range protein standards from 2.5kDa to 200kDa

Protein	Molecular weight
Myosin (rabbit muscle)	200,000 Da
β galactosidase (*E. coli*)	116,300 Da
Phosphorylase b (rabbit muscle)	97,400 Da
Bovine serum albumin	66,300 Da
Glutamic dehydrogenase (bovine liver)	55,400 Da
Lactate dehydrogenase (porcine erythrocyte	36,500 Da
Carbonic anhydrase (bovine erythrocyte)	31,000 Da
Trypsin inhibitor (soybean)	21,500 Da
Lysozyme (Chicken egg white)	14,400 Da
Aprotinin (bovine lung)	6,000 Da

Percoll: silica particles, at a density of 1.120±0.005 g/ml

Protogel: 30 % w/v acrylamide, 0.8 % bisacrylamide

RNAsol B: contains unspecified amounts of guanidine thiocyanate and phenol

Sagatal: pentobarbitone sodium (60 mg/ml)

Transcription buffer (x5) commercial preparation containing: 200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl.

Bronchial epithelial growth medium (BEGM). contains hydrocortisone, epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, and BSA; unspecified amounts